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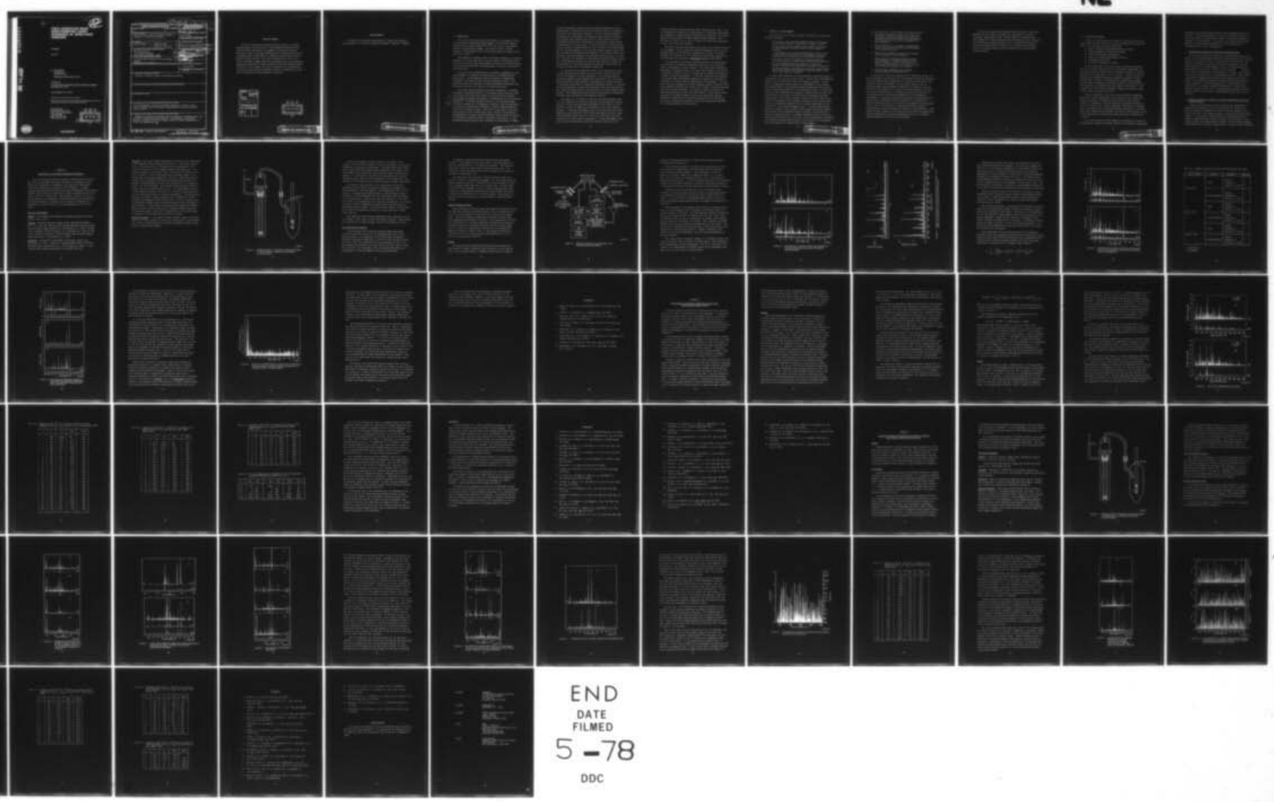

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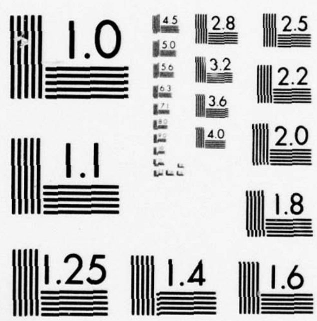

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FIELD IONIZATION MASS SPECTROMETRIC RAPID DIAGNOSIS OF INFECTIOUS DISEASES

Final Report

July 1977

By: Michael Anbar
Kyoung R. Kim
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Mass Spectrometry Research Center

Supported by:

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Using new chemical procedures, mass spectrometric instrumentation, and appropriate computerized data analysis, the diagnosis of infectious diseases, through the molecular weight profile of neutral metabolites in urine, was demonstrated.

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EXECUTIVE SUMMARY

This report describes the fourth and final phase of a research program aimed at the utilization of multicomponent analysis by field ionization mass spectrometry for diagnosis of infectious diseases. During this phase it has been demonstrated that infectious diseases can be diagnosed with a high degree of confidence through analysis of the molecular weight profile of the neutral metabolites in urine. New, faster, and simpler sample preparation techniques, and new, nonrigorous, computerized statistical analysis techniques have been developed during this phase of the program. The results corroborate the conclusions of the previous phase that multicomponent analysis of metabolites in urine can be an effective clinical diagnostic technique.

ACKNOWLEDGEMENT

The authors of this report acknowledge with thanks the assistance of Mr. Gilbert A. St. John in the mass spectrometric analysis of samples.

I. INTRODUCTION

This is the fourth annual and final report on a program on "Field Ionization Mass Spectrometric Rapid Diagnosis of Infectious Diseases" conducted under contract DAMID17-74-C-4047 with the U.S. Army Medical Research and Development Command. This report covers the period December 1, 1976 through July 30, 1977 and concludes the research effort under this contract.

The capability of making a rapid and reliable diagnosis of infectious diseases at an early stage and at low cost would be of especially great value to the military where large numbers of soldiers are often stationed in confined areas and their continuing health is crucial to carrying out their objectives.

Our goal in this program has been to develop a methodology for the rapid diagnosis of infectious diseases based on nonfragmenting mass spectrometry. The experimental approach is based on a methodology developed at SRI using new types of field ionization sources. This methodology allows the detection of specific metabolic aberrations that may occur in the host as a result of an infectious process.

Our overall program comprised three major components: instrument development (sponsored primarily by the National Cancer Institute Grant #1 R01-13312 and the National Institute of General Medical Sciences Grant #DAAG29-76-C-0056), development of methodologies for the preparation and analysis of clincial specimens (urine) for the molecular weight profiles of metabolites of intermediate volatility, and the development of statistical computational techniques to extract maximum diagnostic value from the analytical data. Over the last four years we have made good progress in each of these three areas. We have by now adequate instrumentation to produce reliable molecular weight profiles as well as to determine the chemical nature of individual constituents. We have developed improved and more rapid techniques for preparation of samples

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See last page

for mass spectrometric analysis and have developed computational data handling that facilitates the pattern recognition analysis of metabolites associated with pathological conditions. We have analyzed adequately large groups of urine samples from patients having infectious diseases, have compared these analyses with urine of normal subjects, and have established characteristic differences in the molecular weight profiles of metabolites of the two groups. We have also shown in a demonstration study on metabolic changes induced in rats by lesions in the hypothalamus that one can differentiate by the molecular weight technique between two sets of metabolic aberrations--those induced by the surgical insult (sham operation) and those induced by the localized specific lesions. We have also shown that our technique can successfully distinguish between normals and a heterogeneous population of patients with infectious disease and differentiate out from this population patients with acute urinary infections and those with acute pulmonary infections.

In a parallel effort sponsored by ARO, we have developed new types of field ionization sources which are less prone to deactivation when used for the analysis of acidic substrates. On yet another project sponsored by NIGMS, we have designed and constructed a novel dual-stage mass spectrometric system capable of determining the chemical structure of individual constituents of complex multicomponent mixtures by collision-induced decomposition. We have also developed, under the sponsorship of NCI, on-line computerization of the mass spectra of highly complex organic mixtures. This computer-controlled system facilitates more reproducible and faster mass spectrometric multicomponent analysis.

The results of our efforts of last year have been published under the title "Diagnosis of Infectious Hepatitis by Multicomponent Analysis with Use of Field Ionization Mass Spectrometry" [Clin. Chem. 22, 1503 (1976)], a preprint of which was incorporated in last year's report as Appendix A. Instrument development work on the ionization source which appeared in press under the title "Preactivated Highly Efficient Linear Field Ionization Source" (Rev. Sci. Instr. 47, 1270, 1976) was incorporated in last year's report as Appendix B. Further development along this line has now appeared in Analytical Chemistry under the title

"Glass Lined Field Ionization Sources (Anal. Chem. 49, 2121, 1977). The ion fragmentation technique for identification of individual constituents was described in a paper entitled "Combined Field Ionization - Ion Kinetic Energy Mass Spectrometer for Multicomponent Mixutre Analysis" (Int. J. Mass Spec. Ion Phys. 24, 37, 1977), a preprint of which was incorporated in last year's report as Appendix C.

This report includes three major topics which cover different aspects of our program. The first describes improved methodology and use of new instrumentation for the analysis of metabolites in urine. The second describes the potential use of the molecular weight profile technique in the differentiation between two simultaneous metabolic aberrations.

The improvements in the data handling techniques were demonstrated on the old data of infectious hepatitis as well as on the more recent series of experiments on other patients. The results show a substantial improvement in diagnostic power. The third topic comprises the analysis of metabolic profiles of urines of normals and of patients suffering from a variety of infectious diseases. It has been shown here that comparing a relatively small heterogeneous population of patients to an equal set of normals, the former could be diagnosed with 93% confidence. The diagnostic efficacy increased to 95% when examining a subset of patients with acute pulmonary infections and to 100% with a subset of patients suffering from acute urinary infections. The latter two subsets could also be differentiated from each other with 100% efficacy. These results, which corroborate our former findings in the case of infectious hepatitis demonstrate unequivocally the potential usefulness of our methodology for clincial diagnosis and therefore fulfill our expectations expressed in the original proposal five years ago.

II. SUMMARY OF ACCOMPLISHMENTS

The objective of the proposed program of research is to answer the following questions:

- Do certain or most infectious diseases exhibit characteristic concentration patterns of metabolites in plasma or urine?
- To what extent are the changes in the chemical constitution of these biological fluids indicative of the severity of infection?
- To what degree can the chemical aberrations in urine be used as indicators for recovery, and to what degree can they be useful for the identification of post-infection carriers?
- Do carriers, who do not exhibit any clinical symptoms of infection, produce any significant characteristic metabolites?
- Can microorganisms in minute quantities be identified through a characteristic nonpolymeric biochemical fingerprint?
- Can microorganisms be identified, through their characteristic metabolites released into controlled artificial media, within one to three hours of incubation?

To answer these questions, we have envisaged an extensive program of research that will take five years or more. All the basic instrumentation necessary for the feasibility phases is currently available. We have added a fragmentation chamber setup and a minicomputer to our systems. The advanced phases of the proposed research program would thus benefit from the augmented capabilities of our analytical systems. At this stage, however, we are terminating the program of research after less than four years because of the transfer of the principal investigator from SRI International to the School of Medicine, State University of New York at Buffalo. Nevertheless, this premature termination of this program leaves it with well documented evidence that the proposed methodology has the potential of a very powerful diagnostic tool. To explore these assumptions, we proposed to test different urine extracts from normal subjects, from patients with established infectious diseases, and from the same patients following recovery. If chemical fingerprints were to be determined in different extracts of the urine, and if characteristic aberrations were observed, attempts would be made to select methods of fractionation that would enchance the demonstration of these changes.

In the fourth year of this program, our objectives were:

1. Investigate the metabolic profiles of urine from patients of infectious diseases other than liver infections and find out whether our methodology has more general applications than the diagnosis of liver disorders.

2. Find out whether it is possible to differentiate between two disorders contributing simultaneously to the metabolic profile.

3. Improve and streamline the analytical procedure and to ascertain its reproducibility.

4. Improve the data handling procedures and make them independent of arbitrary decisions of the analyses, such as the cut-off point of the p value in the WNI analysis or the threshold point in the differential between two classes.

5. Apply the same techniques to the analysis microorganisms and their metabolites.

We have met the first four objectives with unequivocal success, but have not managed to proceed with the fifth objective within the limitations of the shortened project (termination of the project by the end of July instead of the end of November). We have shown, however, that the molecular weight profile diagnostic method is not limited to liver disorders and can be applied to a variety of infectious diseases. We have also shown potentially that if two metabolic aberrations contribute to an abnormal pattern they can be differentiated by our methodology. Furthermore, in a parallel research effort sponsored by Edgewood Arsenal under contract #DAAA-76-C-0135 we have shown that by analyzing the molecular weight profiles of just purines and pyrimidines from hydrolized nucleic acids it was possible to differentiate between a number of microorganisms in spite of substantial variance in the methodology. These results strongly suggest that the molecular weight profile technique when applied simultaneously to many more metabolites will be an extremely powerful diagnostic tool for the microbiologist.

The mass spectrometric multicomponent analysis seems to excel other metabolic profile analysis techniques for two reasons; it provides information simultaneously about a larger number of constituents and this information is more reproducible than attainable by other techniques. More differential diagnosis studies as well as longitudinal studies on the same individuals are now called for to corroborate and extend the scope of the highly promising results obtained in this foreshortened research program.

III. TECHNICAL BACKGROUND

In order to avoid repetition, we shall not include in this section topics which have been covered in the last two annual reports (1975 and 1976). These include the sections describing:

A. Chemical Diagnosis through Multicomponent Analysis

B. Chemical Diagnosis of Infectious Diseases

C. Field Ionization Mass Spectrometry

D. Analysis of Complex Multicomponent Mixtures

E. Instrumental Development

F. New Sample Preparation Procedures

Section E described in last year's report (Technical Background, Section A) includes practically all the instrumental development with the exception that we adapted lately a different technique for growing dendrites on the sharp edge of our slit sources. Instead of using the lengthly high temperature carbonaceous dendrite technique of Beckey we adapted (as a result of a research effort sponsored by ARO under contract #DAA-27-76-0056) an electrolytic deposit of cobalt dendrites. This technique is described in the Final Report of that project. Next we improved on the source's performance, reducing its memory by glass lining. (Anal. Chem. 49: 2121, 1977).

Section F described in last year's report (Technical Background, Section B) included practically all the innovation in the sample preparation procedures an updated description with a few new data are presented in Appendix A. The most important information in Appendix A is the fact that we have in hand a reasonably fast analytical technique with an acceptable level of precision (average coefficient of variation about 10%). Inasmuch as we determine the relative concentrations of close to 200 compounds with this precision within two hours, our methodology is unique.

In the next sections we shall summarize the results of our study of differentiation between the abnormal patterns of two metabolic aberrations

coexisting in the same biological system. This was demonstrated on rats as models. We shall then describe in brief our findings on analyzing urines from patients suffering from a variety of infectious diseases in addition to liver infections. In the last part of this Technical Background we shall discuss the ramifications of our findings and our recommendations for future investigations.

A. Differentiation Between Two Coexisting Abnormal Patterns

Urine from rats which have undergone an operation inflicting specific lesions in their hypothalamus (Group A) and rats undergoing sham operations (Group B) was compared to urine of control intact rats (Group C). The results are described in detail and discussed in Appendix B. We have shown that each of the three groups A, B, and C are significantly distinguishable from each other. Moreover, we can recognize differentiate a pattern characteristic of the operation (five weeks after surgery) and a pattern characteristic of the hypothalamic lesions. Group A shows a combination of both patterns whereas Group B shows only the former pattern. This study was performed on an animal model to guarantee controlled conditions. The results demonstrate a very important point--namely that if we encounter metabolic aberrations in a subject which originate from two sources, (e.g., a general fibril state and a specific infective agent) we may be able to differentiate between them with our technique.

B. Diagnosis of Different Infectious Diseases Through Molecular Weight Profile Analysis

During the previous phase of this program we have demonstrated a highly effective diagnosis of infectious hepatitis by analyzing the molecular profiles of metabolites in urine. This was, however, an expected result since liver damage is known to induce far-reaching metabolic changes. An open question remained whether our methodology would be capable of diagnosing other infectious diseases that are likely to manifest themselves in profound metabolic changes. We have therefore examined a mixed population of patients suffering from a variety of acute infectious

diseases, including patients with acute urinary infections and patients with acute pulmonary infections as subsets. The results are presented and discussed in Appendix C.

We have shown here that we can differentiate the total population of acute infections from the normal population with 93% success, the subset of urinary infections with 100% success and the pulmonary infections with 95% success. These are highly encouraging results, especially when one takes into account the relatively small populations examined. Two factors have contributed to these impressive results; the high reproducibility of the technique (Appendix A) and the improved statistical analysis.

c. Conclusions

In this research program we have shown that field ionization mass spectrometric molecular weight profile analysis is a clinical analytical tool with the potential of being a very useful routine diagnostic tool. This technique has been shown to differentiate acute liver, urinary, and pulmonary infections, as well as differentiating a heterogeneous population of patients with infectious diseases from healthy subjects. It was also shown that if two metabolic aberrations coexist in a given subject the two abnormal patterns can be differentiated from each other. It is believed that the same principle can be applied to three or more coexisting patterns.

The next steps in this research program, when it is continued, should include:

(a) Longitudinal studies on the same subjects prior to, during, and following the acute infection. This may also provide information on the possibility of identifying "healthy" carriers of the infection.

(b) Determination of the chemical identity of constituents, the concentration of which is significantly changed under pathological conditions.

(c) Establishment of the scope and limitation of the same technique in determining molecular weight profiles in plasma and saliva.

(d) Establishment of the potential use of the technique to identify microorganisms directly or via their excreted metabolites.

APPENDIX A

Improvements in the Sample Preparation Procedures

The previously used isolation procedure[1] required several pH adjustment steps and treatment with urease overnight, thus making it rather lengthy and complicated. Furthermore, two consecutive concentration steps after isolation were required for this technique. A simpler sampling method employing fractionating column chromatography was developed, therefore, which allows the isolation of organic substances from the biological matrix in a single step. This technique is similar to one previously applied to the sampling of volatile metabolites for GLC analysis.[2] The present technique is suitable for the analysis of metabolites of intermediate volatility (10^{-4} Torr at room temperature to 10^{-4} Torr at 200°C).

Materials and Methods

Samples. Urine samples were obtained from healthy volunteers and were stored frozen (-17°C).

Reagents. Methylene chloride, reagent grade, (Mallinckrodt Chemical Works, St. Louis, MO, USA) was distilled from phosphorus pentoxide, Baker Analyzed Reagent (Baker Chemical Co., Phillipsburg, N.J., USA). Diethylether, anhydrous, (Fisher Scientific Co., Pittsburgh, Pa., USA) was used without further purification. Sodium chloride, Baker Analyzed Reagent, (Baker Chemical Co., Phillipsburg, N.J.., USA).

Adsorbents. Chromosorb P, 80/100 mesh, acid washed, (Supelco Inc., Bellefonte, Pa., USA). Alumina F-1, Chromatographic Grade, 45/60 mesh, (Applied Science Laboratories, Inc., State College, Pa., USA). Porasil E, 80/100 mesh, (Waters Associates, Inc., Milford, MA, USA).

Apparatus. The urinary organic metabolites were isolated and concentrated by the sampling system illustrated in Figure A-1. This system consists of two custom made parts: an "isolator" and a "concentrator". The isolator is made from a 19/20 standard tapered pyrex glass joint. The isolation column is a glass tube (135.0 x 8.0 mm O.D., 6.2 mm I.D.) with one end rounded. It was firmly packed with an adequate amount of adsorbent and its ends were plugged with glass wool. Before and after the packing, both the adsorbent and glass wool were carefully washed with methanol, dichloromethane, and ether and then baked out in a vacuum oven (200°C, 10^{-1} Torr) overnight. The isolation column was then stored in a culture tube with a Teflon-lined screw cap. The concentration column is a glass capillary (18.0 mm x 2.0 mm O.D., 1.4 mm I.D.) which easily fits into the solid sample holder of the mass spectrometer.[3] The concentration column was prepared by tightly packing 15 mg of chromosorb P into the capillary and plugging the ends with glass wool. Prior to packing, both the adsorbent and glass wool were washed and backed in the same manner as described above. After packing, the concentration columns were rebaked under vacuum for 48 hours, and stored in a glass container sealed with a Teflon-lined plastic screw cap. The preparation of the columns takes an average of 30 min/column when carried out in batches.

Sampling Procedure. A 1 ml sample of urine (neutral, acidic, or alkaline) and 0.3 g of sodium chloride are placed in the outer tube of the isolator, and a drop of concentrated HCl, or one drop of 6N NaOH, is added to make acidic or alkaline urine samples.

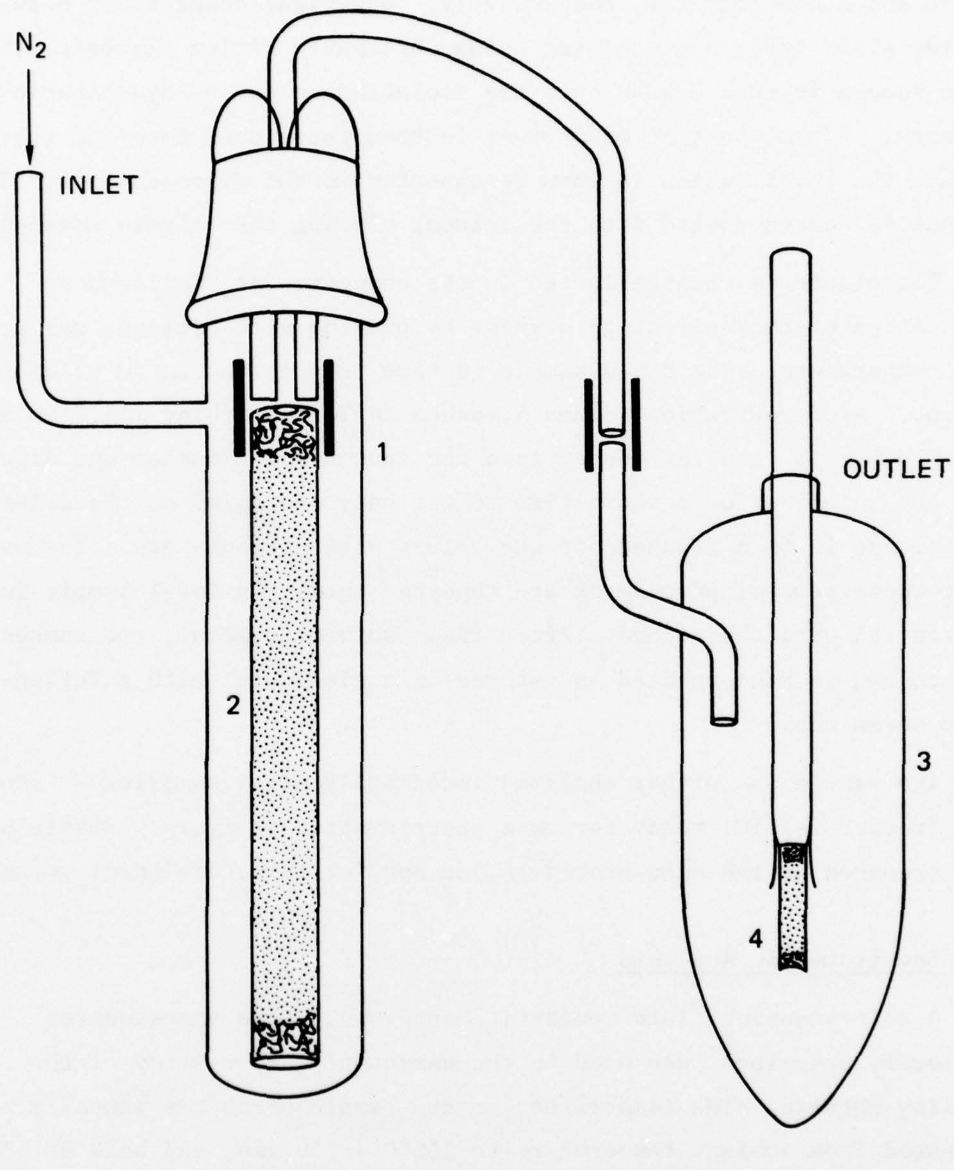

FIGURE A-1 SAMPLING SYSTEM: (1) ISOLATOR, (2) ISOLATION COLUMN, (3) CONCENTRATOR, (4) CONCENTRATION COLUMN, (5) TEFLON TUBING

After vortex mixing, the tube is fitted to the jacket of the isolator, the inlet and the outlet of which are attached to an isolation column and a concentrator, respectively. Gas-tight connections between the two glass tubes are achieved using shrinkable Teflon sleeves. The urine sample is then loaded onto the isolation column by hydrostatic N_2 pressure. A 5-ml portion of solvent is then introduced into the tube through the inlet, which is then reconnected to the nitrogen line. The solvent is forced upward into the column, eluting the organic metabolites.

The eluate is then collected in the concentrator, followed by evaporation of the solvent to dryness by purging with nitrogen gas at room temperature. The dried sample is then redissolved in 50 μl of the solvent. A concentration column attached to Teflon tubing (11.0 mm x 4.0 mm O.D.) is then introduced into the concentrator outlet and dipped into the solution for a short time to wet only one-third of the column. The solvent is then flushed off the column with nitrogen gas. The wetting and solvent removal procedures are repeated until the total sample is transferred onto the column. After final solvent removal, the concentration column is disconnected and stored in a clean vial with a Teflon-lined screw cap.

The sample was either analyzed immediately after sampling or stored in a freezer (-17°C) ready for mass spectrometric analysis. Sample blanks were prepared by the same procedure, except for the addition of urine.

Mass Spectrometric Analysis

A multiscanning field ionization quadrupole mass spectrometer previously described[1] was used in the semiquantitative study of the sampling methods. The temperature of the sample probe was manually increased from ambient temperature to 160°C in 30 min. and held at 160°C for 10 min. while the ionization source was maintained at 220°C. The mass analyzer was scanned over the mass range of m/e 60 to 260 at a rate of 16 sec/scan. This instrument, which has limited sensitivity, was superseded by a 35-cm, 60° magnetic sector mass spectrometer.

The magnetic multiscanning field ionization mass spectrometer (Figure A-2) was employed in the later stages of the investigation to study the reproducibility of the analytical procedure. The ionization source was a slit type[4] glass-lined[5] cobalt-activated source. The electrolytic cobalt-activation was carried out generally following the procedure of Rechsteiner et al.[6]

The sample probe was inserted at -37°C and heated to 180° over a period of 40 min by a temperature programmer. The source temperature was manually adjusted from 100°C to 200°C during the run, keeping it always at a temperature higher than that of the sample probe. The mass range of 86 to 304 amu was scanned in 16 sec. The multiscanned spectra were collected by a multichannel analyzer (Nuclear Data ND 2400) and stored in digital form (4096 words) on a nine-track magnetic tape (Figure A-2). The data were then transferred to, and analyzed by, a Burroughs 6700 computer.

Computer Analysis of Data

After smoothing by the least-squares procedure of Savitzky and Golay, the data were processed as described earlier,[1] with the exception that the spectra were normalized to unit area for all but the five largest peaks. This avoids variances determined by variations in peak areas whose magnitudes would dominate the values of the normalization constants. The reproducibility of the entire analytical procedure was evaluated by computing variances for the normalized peak areas at each mass number. The value obtained by averaging the variances over all reliable mass numbers was used as a figure of merit. A mass number was defined as "reliable" when it had a detectable peak associated with it in all of the spectra comprising the data set.

Results

The sampling device described above provides both isolation and concentration of organic substances from urine matrix in less than one hour. This new method extracts a somewhat different group of organic

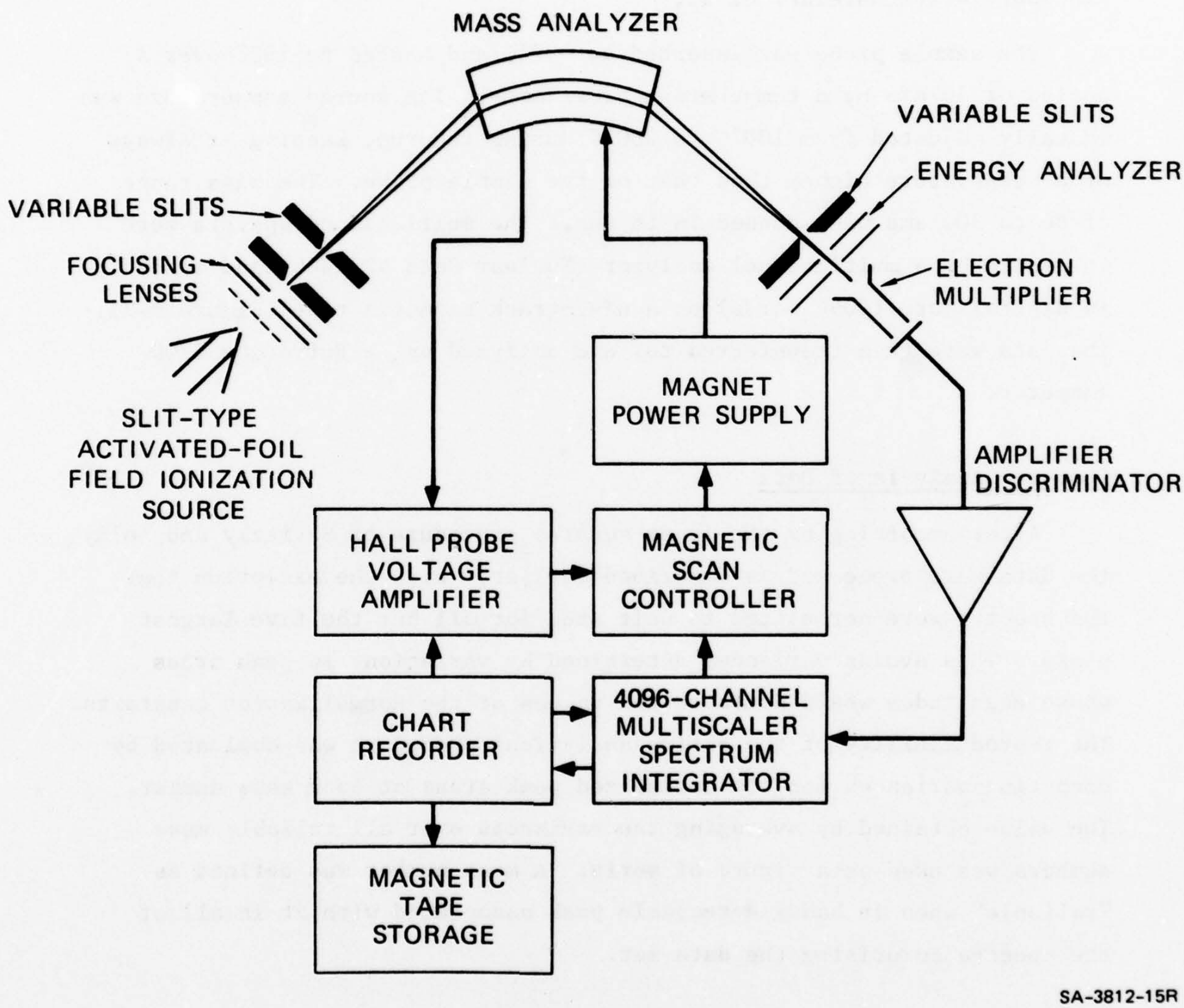

FIGURE A-2 SCHEMATIC DIAGRAM OF MULTISCANNING FIELD IONIZATION MASS SPECTROMETER

metabolites from those obtained by the XAD-2 resin extraction method,[1] as demonstrated by Figure A-3.

The concentrator (Figure A-1) permits both concentration and complete transfer of samples about ten times faster than the previously used capillary evaporation method.[1] The efficiencies of these two methods for identical samples are compared in Figure A-4. As can be seen, the two profiles are remarkably similar, but the new method provides much better recovery. It has been demonstrated that the concentration columns do not introduce significant artifacts, irreversible adsorption, or uncontrollable pressure increase during the mass spectrometric analysis.

Adsorbents other than chromosorb P, such as microglass beads, anachrome Q, and porous glass, have been tested as concentrating agents, but chromosorb P was found to be superior, both in adsorption and recovery. It was noticed that concentrated samples could be stored for quite long periods without apparent losses or changes, as illustrated by Figure A-5.

The optimization of the sampling method required a thorough investigation of three variables, type of adsorbent, the eluting solvent, and the pretreatment of the urine. The proper adsorbent should be nonextractable by the eluting solvent, but should retain the urine, water, urea and inorganic salts, as well as biopolymers, if present, while allowing organic substances of interest to be eluted. Thus, inorganic hydrophilic adsorbents are the most suitable column materials for this purpose. The solvent should preferably have high volatility, high solvent power, water immiscibility, and long-term stability. Alumina, chromosorb P, and porasil E were tested as adsorbents, and ether and dichloromethane as the best solvents.

The optimum amount of adsorbent depends on its loading capacity, the size of the sample, and the volume of eluting solvent. These parameters were experimentally tested and optimized: for 1 ml of urine and 5 ml of solvent, the optimum amount of alumina, porasil E, and chromasorb P were about 3.5 ml, 3.0 ml, and 3.7 ml respectively.

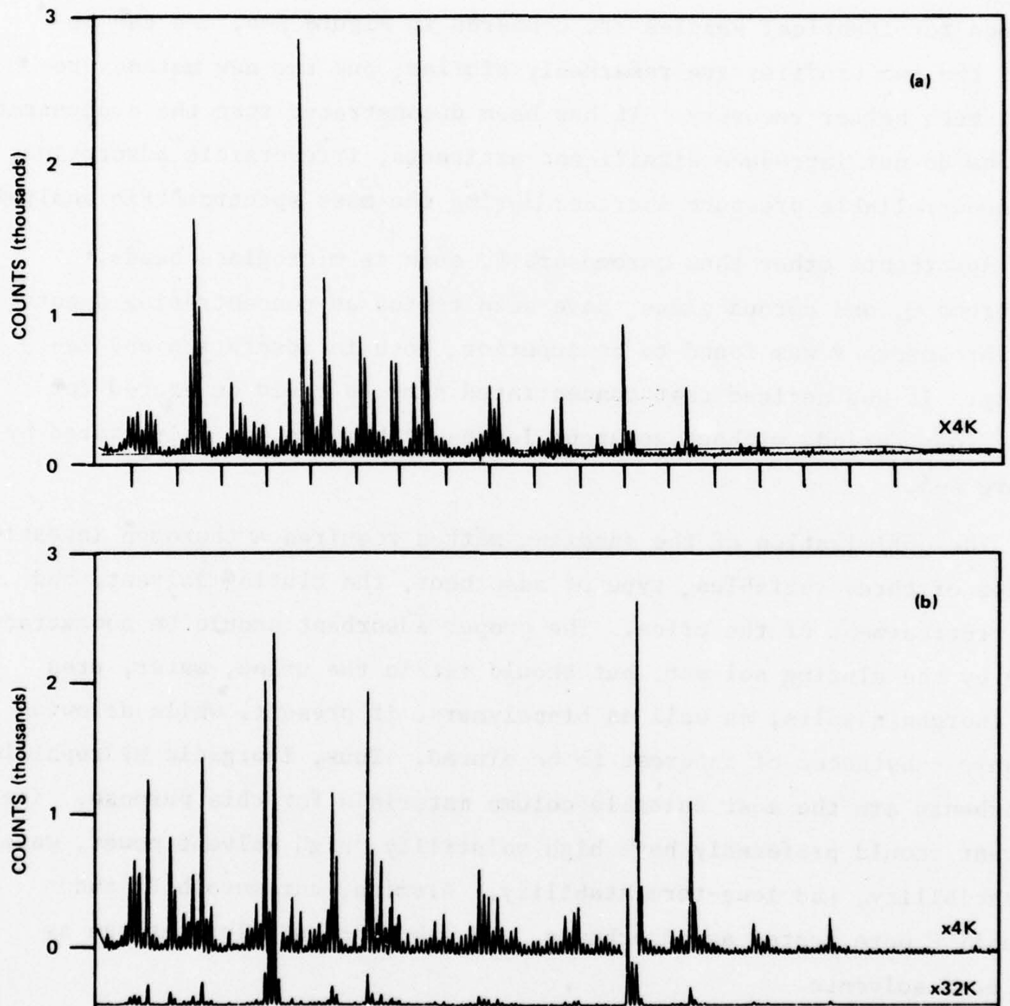

FIGURE A-3 MASS PROFILES OF NEUTRAL METABOLITES OBTAINED BY (a) XAD-2 RESIN EXTRACTION AND (b) THE PRESENT SAMPLING METHOD

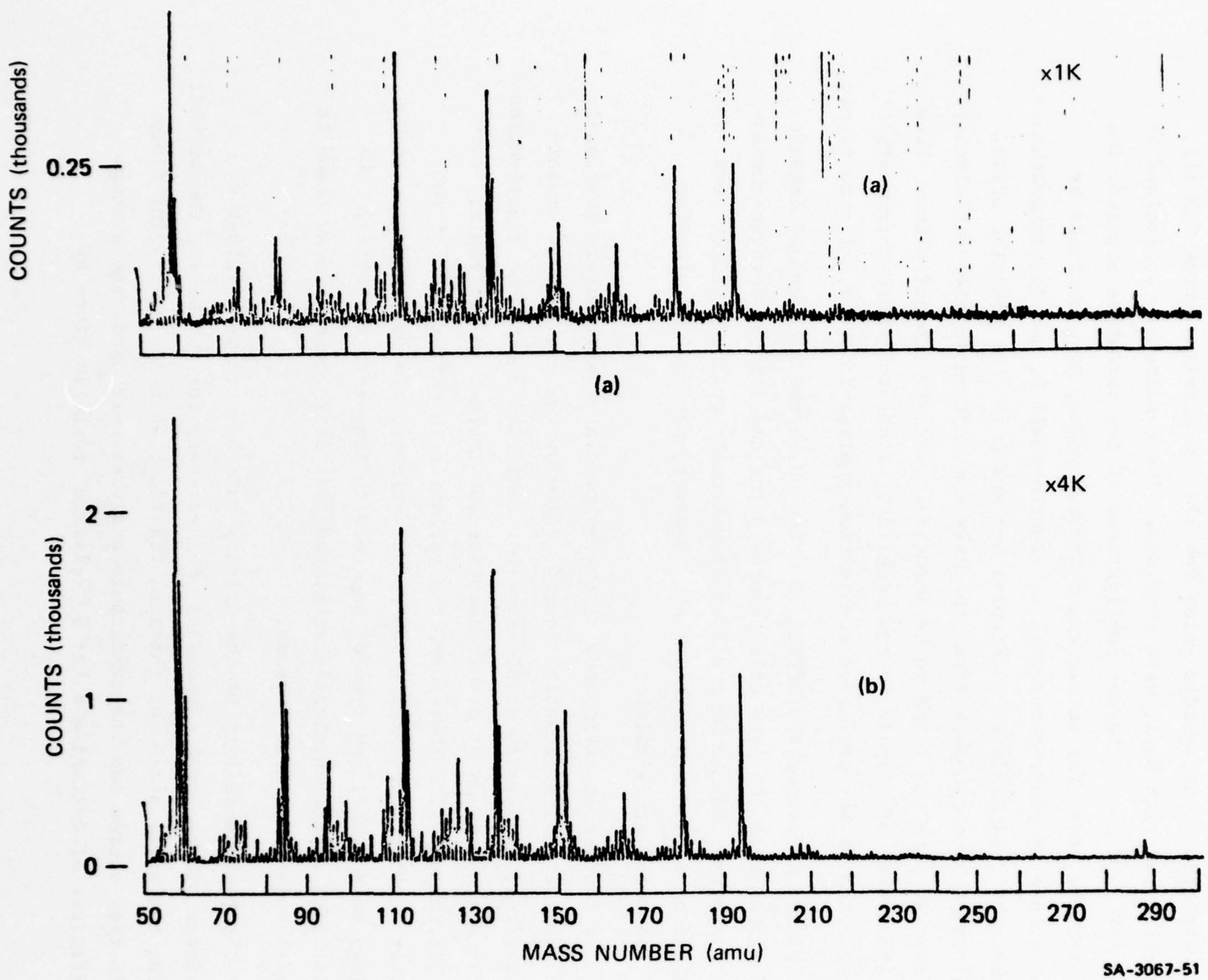

FIGURE A-4 MOLECULAR MASS PROFILES OF NEUTRAL METABOLITES IN A NORMAL URINE SAMPLE (a) OLD METHOD (b) COLUMN CONCENTRATION METHOD

Untreated urine, acidic urine (pH 1), and alkaline urine (pH 11), each saturated with NaCl, were examined. The results are summarized in Table A-1. Of the different combinations of the variables studied, the best results, both for acidic and untreated urine, were obtained by combinations of ether-chromosorb P, ether-porasil E, and dichloromethane-chromosorb P, as depicted in Figures A-6 and A-7. For alkaline urine, dichloromethan-chromosorb P was the only combination to give satisfactory yields. Alumina was found to be inadequate for any type of urine. From the standpoint of simplicity and stability, dichloromethane-chromosorb P was selected as the preferred combination applicable under all conditions.

Figure A-9 shows the effect of urine pH on the recovery of organic metabolites from the same urine sample using the dichloromethane-chromosorb P system. As expected, different molecular weight profiles were obtained at acidic and alkaline pH. However, neutral and alkaline profiles were very similar.

The ionization efficiency of the multipoint field ionizer previously used[1] was very significantly reduced following the analysis of organic metabolites, especially acidic samples. This was due to the deactivation of the source caused by electronegative substrates, in particular, carboxylic compounds.[4] Therefore, the variation in performance of the ionizer provided the largest source of variance. To overcome this shortcoming, we sought a new type of source with long-term stability. The glass-lined electrochemically activated foil slit source[4,5] was found to be well suited for this purpose.

The reproducibility of the urinary mass spectra was evaluated. Knowledge of the error components was necessary for optimizing the method. During the early stages of the investigation, when the multipoint field ionization source and the quadrupole analyzer were used, the average coefficient of variation F for T "reliable" peaks is given by

$$F = \sum_{j=1}^{T} \sum_{j}^{n} \left\{ \left[(X_i - \bar{X}_j)^2 \Big/ (n-1) \right]^{1/2} / \, \bar{X}_j \right\} \Big/ T$$

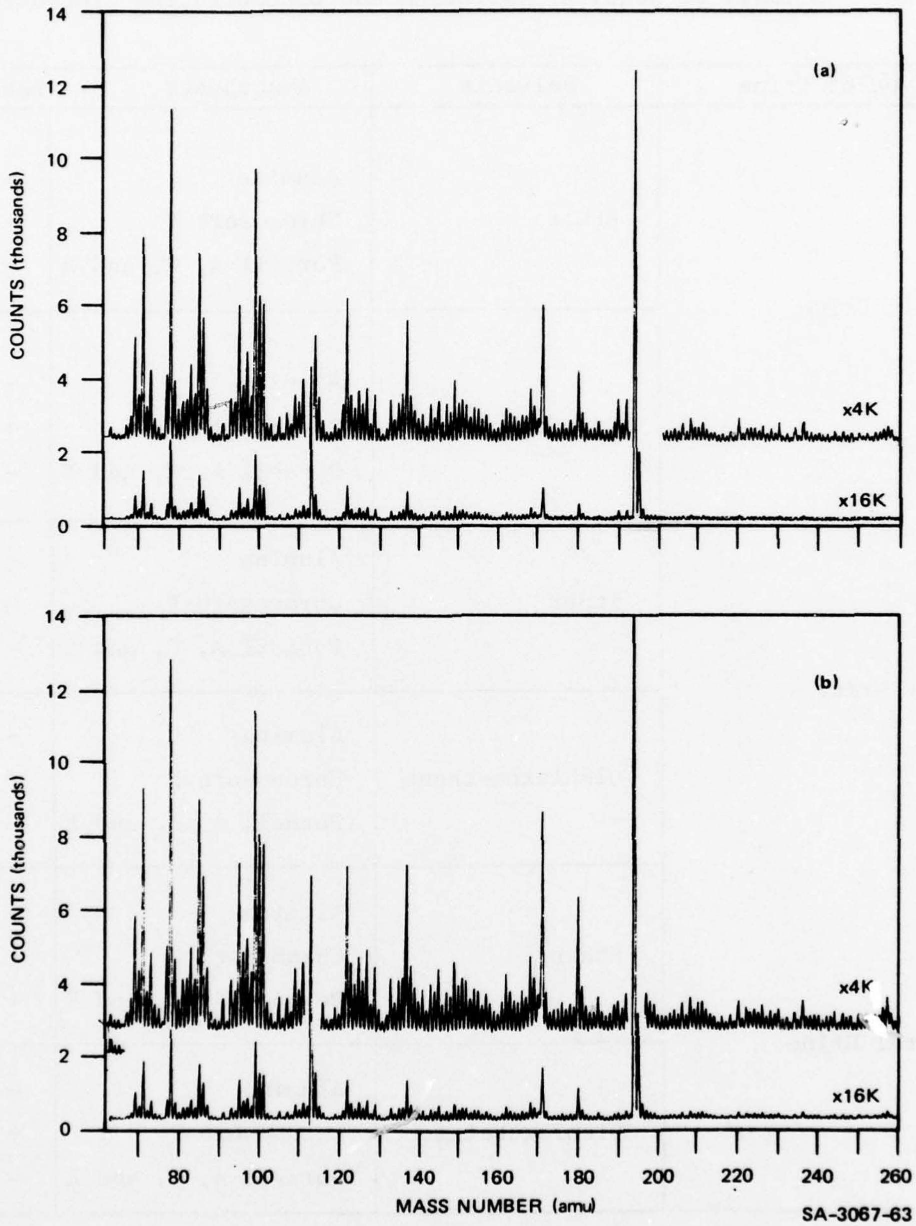

FIGURE A-5 MASS PROFILES OF METABOLITES FROM ALKALINE URINE ANALYZED (a) IMMEDIATELY AFTER AND (b) ONE MONTH AFTER THE PREPARATION

Table A-1. Summary of Results--Isolation and Concentration Procedures

Type of Urine	Solvents	Absorbents	Results
Acidic Urine	Ether	Alumina	–
		Chromosorb P	+
		Porasil A, C, and E	+
	Dichloromethane	Alumina	–
		Chromosorb P	+
		Porasil A, C, and E	–
Basic Urine	Ether	Alumina	–
		Chromosorb P	–
		Porasil A, C, and E	–
	Dichloromethane	Alumina	–
		Chromosorb P	+
		Porasil A, C, and E	–
Neutral Urine	Ether	Alumina	–
		Chromosorb P	+
		Porasil A, C, and E	+
	Dichloromethane	Alumina	–
		Chromosorb P	+
		Porasil A, C, and E	–

+ = Satisfactory

– = Inadequate

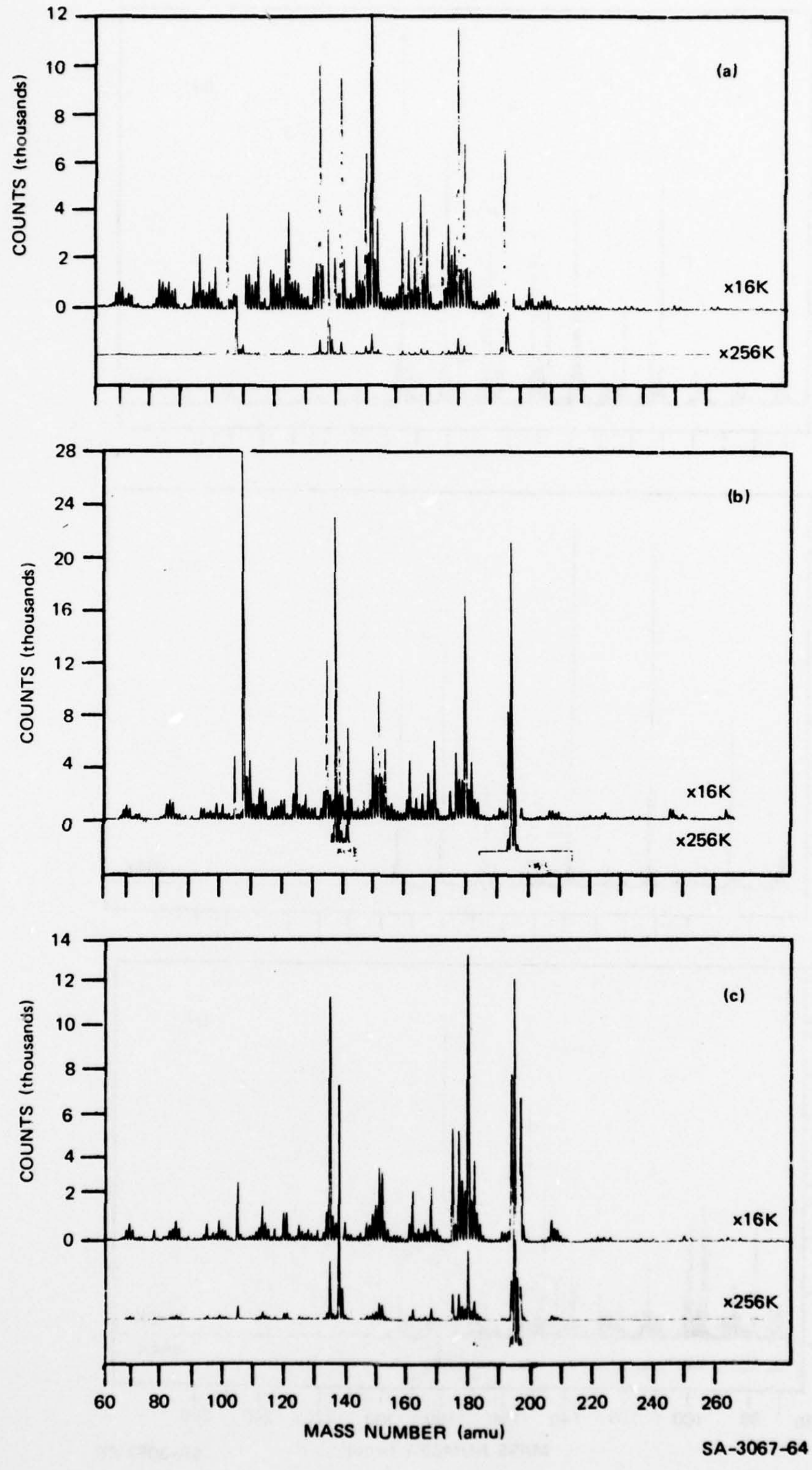

FIGURE A-6 MASS PROFILES OF ALKALINE URINE OBTAINED BY COMBINATIONS OF (a) ETHER-PORASIL E, (b) ETHER-CHROMOSORB P, AND (c) DICHLOROMETHANE-CHROMOSORB P

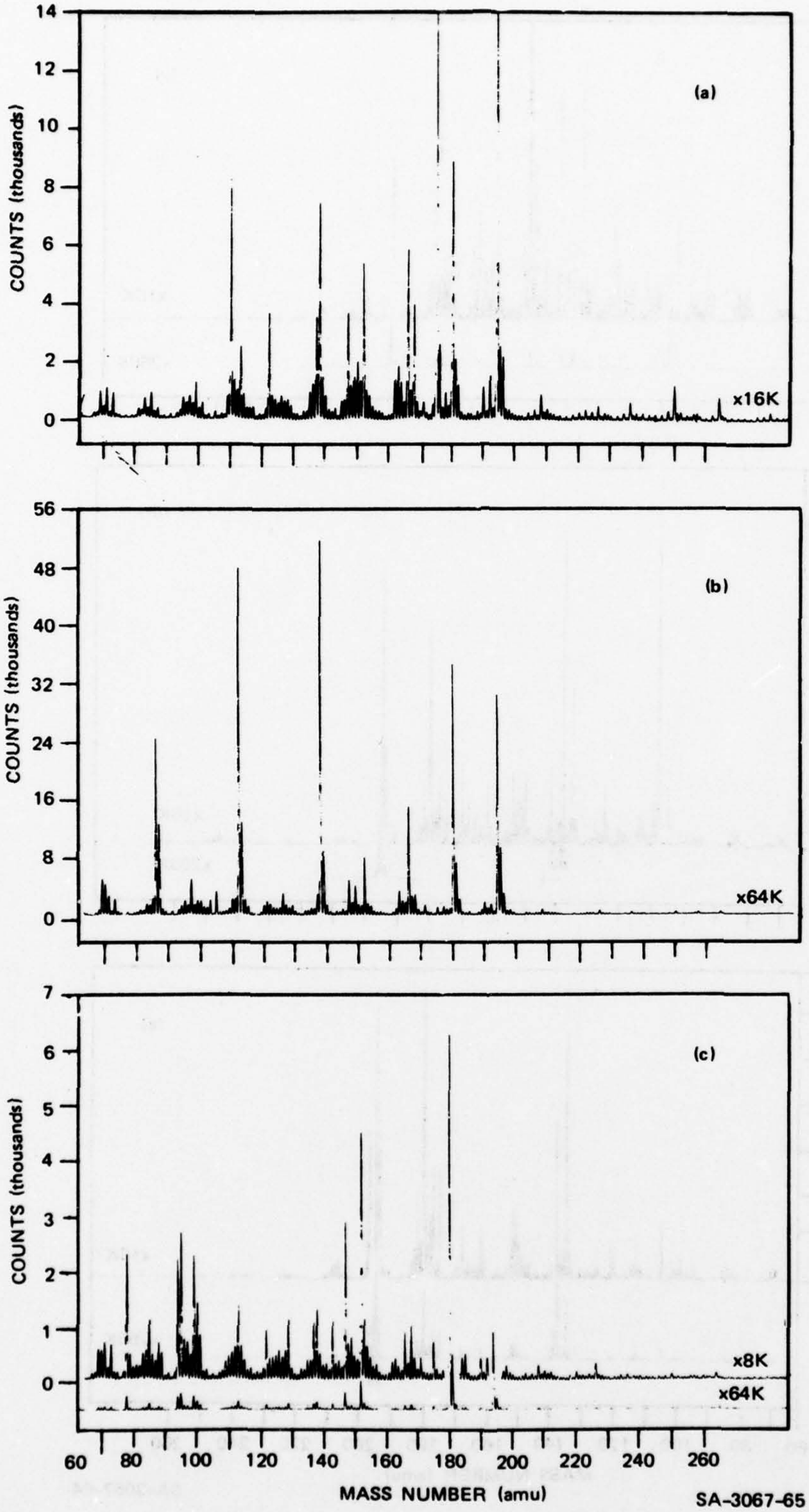

FIGURE A-7 MASS PROFILES OF NEUTRAL URINE OBTAINED BY COMBINATIONS OF (a) ETHER-PORASIL E, (b) ETHER-CHROMOSORB P, AND (c) DICHLOROMETHANE-CHROMOSORB P

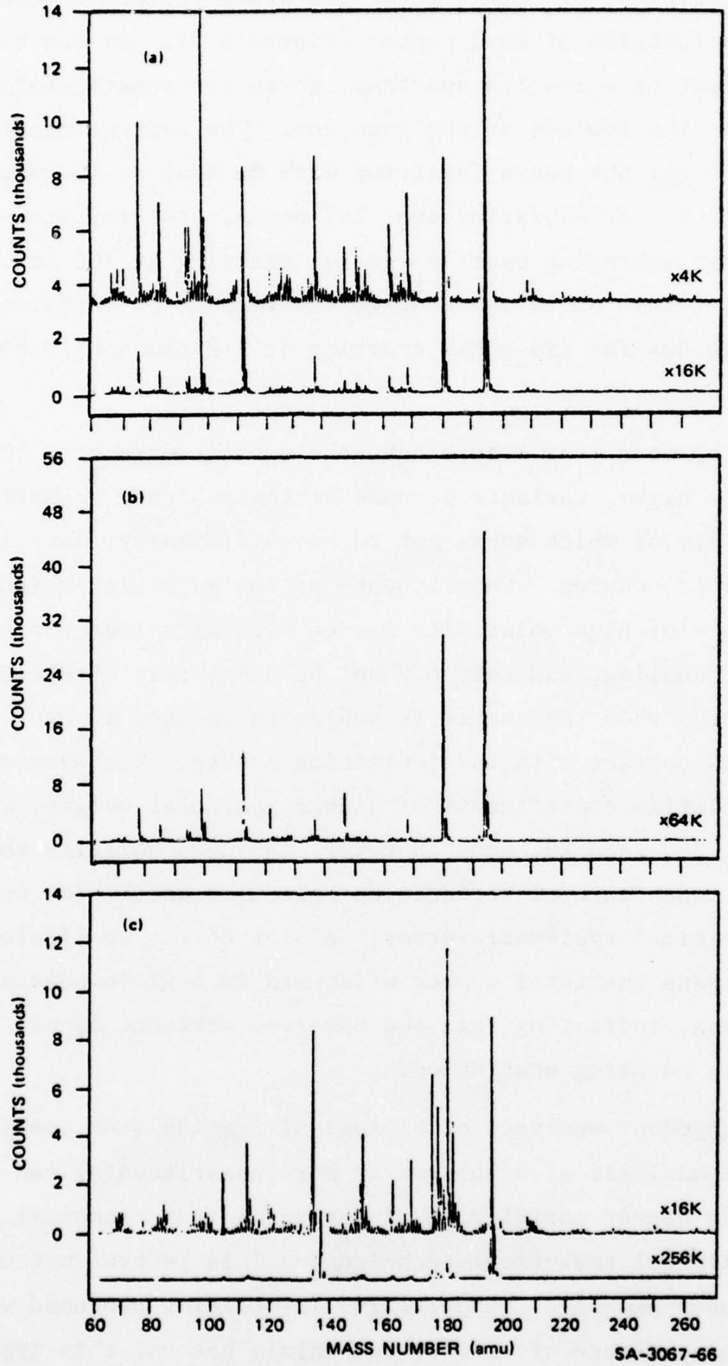

FIGURE A-8 MASS PROFILES OF METABOLITES OBTAINED BY DICHLOREMETHANE-CHROMOSORB P COMBINATION FROM (a) ALKALINE URINE; (b) NEUTRAL URINE; AND (c) ACIDIC URINE

The coefficient of variation of the areas of each of these peaks was plotted as a function of mass number (Figure A-9). As can be seen from the coefficient of variation spectrum, there are substantially larger variations at the low end of the spectrum. The average coefficient of variation for all the peaks (starting with 86 amu) is 15.18%. This value drops to 11.56% when averaging over 187 peaks, starting at 96 amu, and to 11.18% when averaging over 183 peaks, starting at 102 amu. No significant further reduction in the average coefficient of variation is observed (we obtain 10.68% for 175 peaks starting at 110 amu and 10.83% for 156 peaks starting at 130 amu).

As can be seen from Figure A-9, the constituents of < 100 amu show significantly higher variance because of their higher volatility, the reproducibility of which seems not to be sufficiently controlled by our experimental procedures. Constituents of low molecular weight and therefore generally of high volatility may be partially lost during the sample storage and handling, and they may not be completely retained during the precooling step when the sample is subjected to vacuum prior to eventual insertion and contact with the ionization source. The same may be true of rather volatile constituents of higher molecular weight, such as those at 193, 221, 260, and 294 amu. However, the less volatile constituents show little dependence of variance on molecular weight (or volatility), indicating minimal systematic error. A plot of the coefficient of variation versus the total number of counts in a given peak area showed no correlation, indicating that the observed variance is not due to limitation by counting statistics.

Multicomponent analysis of biological samples (the simulatneous quantitative analysis of a hundred or more constituents) can tolerate substantially higher coefficents of variation than classical single component clinical analytical techniques. This is true not only because of the enormous amount of quantitative information produced with a reasonable expenditure of effort, but mainly because this type of analysis aims at (1) the detection of patterns, i.e., the simultaneous changes of a number of constituents, and (2) the detection of hitherto unknown changes in the concentrations of individual constituents associated with a given

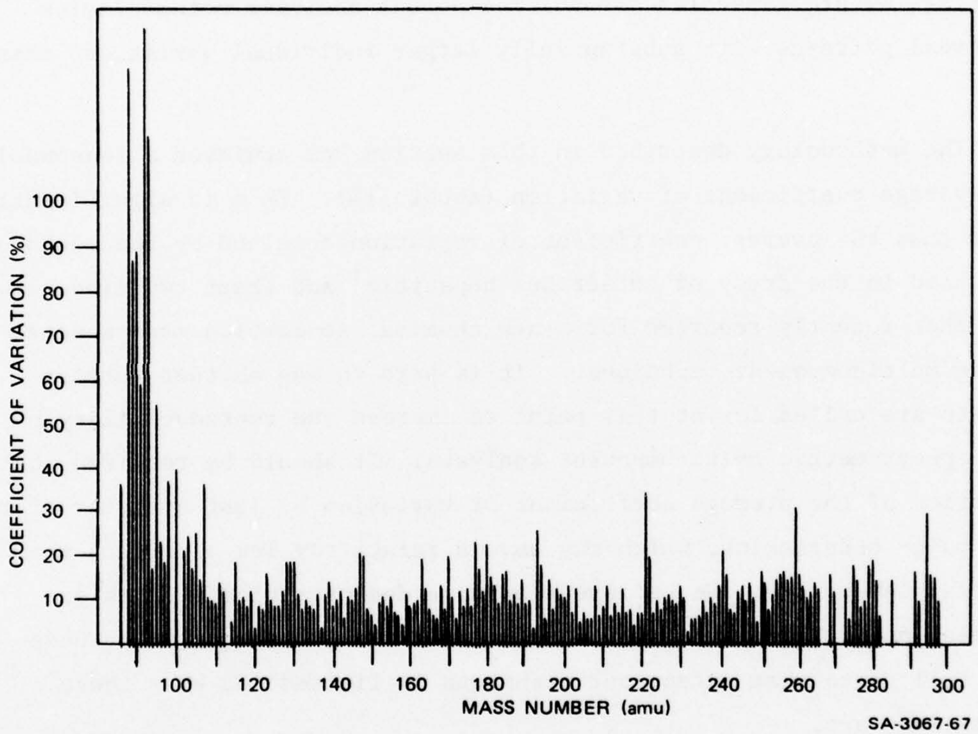

FIGURE A-9 DISPLAY OF THE COEFFICIENT OF VARIATION AS A FUNCTION OF MOLECULAR WEIGHT (COMPUTED FROM NINE CONSECUTIVE D DETERMINATIONS OF THE SAME SAMPLE)

physiological or pathological state which exceed the intrinsic biological variation. In other words, if, for instance, a given pattern involves ten constituents, five of which increase in concentration by just 10% and the other five decrease by the same small percentage, one can still establish its presence, even if the coefficient of variation for each constituent is 10%, since the probability that the random variance will coincidentally follow the sequence of the pattern is rather small. This makes the pattern detectable in spite of the variance. Now, generally speaking, we are expecting the multicomponent analysis methodologies to reveal patterns with substantially larger individual variations than 10%.

The methodology described in this section has achieved a reasonably low average coefficient of variation (about 11%). This is significantly lower than the average coefficient of variation obtained by the methodology used in the study of infectious hepatitis[1] and about two times lower than that recently reported for a new chemical ionization mass spectrometric multicomponent technique.[7] It is hard to say whether further efforts are called for at this point to improve the reproducibility of mass spectrometric multicomponent analysis. It should be realized that reduction of the average coefficient of variation by just a factor of two is a major undertaking, which may have a relatively low payoff in the clinical laboratory. We believe that more demonstration studies on clincial applications of these methodologies are now required. These will tell us to what extent our technique is limited and what these limitations are.

The results of the statistical analysis indicate that the multicomponent analytical technique described in this paper is quite promising when aimed at diagnosing a metabolic disorder on the basis of a characteristic pattern of a subset of constituents in its molecular weight profile. It will identify the metabolites of interest by their molecular weights, and thereby opens up the possiblity of structural identification by secondary collisional fragmentation analysis.[8]

It may be concluded that the application of FIMS multicomponent analysis is not limited by the level of effort required or by the precision of the technique. However, it will take a number of careful clinical studies, such as the one on infectious hepatitis[1] (which has not yet benefited from these recent developments of the technique), to establish its usefulness for biomedical research and clinical diagnosis.

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APPENDIX B

Manifestation of Metabolic Aberrations in Rat Urine Following Hypothalamic Lesions

Different metabolic and endocrine disturbances have been induced by electrolytic injury on different areas of hypothalamus.[1-7] A particular selective bilateral injury[5] of the ventromedial nucleii, the fornices, and medial portions of the lateral hypothalamic areas in the rat is followed by a chronic, diet-sensitive hypercholesterolemia without increase of plasma triglycerides. The pathogenesis of the hypercholesterolemia was investigated and a slow removal of cholesterol from the blood stream and retardation of conversion of cholesterol to bile acid was observed.[8-13]

The hypothalamic-lesion alters hormonal interrelationships, and may be expected to result in significant changes in concentrations of a large number of constituents in body fluids; however, to date only changes in blood plasma or serum have been investigated, and those noted have been confined to alterations in hormone and lipid levels.[8-17] Similarly, changes observed by other investigators after inducing patterns of hypothalamic injury different from ours, are also confined to changes in plasma levels of proteins,[18] urea,[16,19] sodium,[20] and glucose.[16,21]

Hitherto, metabolite excretion has not been investigated in rats with hypothalamic lesions. This may well have come about because the formidable number of metabolites makes investigation by classical methods burdensome. Multicomponent analysis is a method exactly adapted to this difficulty. Multicomponent analysis of metabolites has been widely used in the biomedical field,[22-27] yet no attempt has been made to use this approach to study the chemical composition of body fluids in hypothalamus-injured rats. It seemed of interest, therefore, to measure the concentrations of low molecular weight metabolites using a methodology recently developed in our laboratory for the rapid analysis of complex biological samples.[28,29]

This methodology includes column chromatography for sample preparation, field ionization mass spectrometry (FIMS) for multicomponent analysis,[30] followed by previously described computer data analysis.[29] The objective of the present work was to look for differences in the metabolic profiles of urines from hypothalamus-injured, sham-operated, and intact, control rats. The results indicate distinctive arrays of metabolic changes associated with the hypothalamic lesion.

Methods.

Thirty male Long-Evans rats (10 weeks old, 260-340 g weight) were randomly divided into three groups. Ten rats (group A) received two hypothalamic electrolytic lesions on each side, according to the previously published procedure.[13] This consists of passing a current of 2 mA for 10 seconds through electrodes placed 1.4 and 1.8 mm, respectively, posterior to bregma, 0.75 mm lateral to the midline and 9.5 mm beneath the surface of the brain. It may be noted that these coordinates suffice only for Long-Evans strain rats. Other strains, ACI for instance, require a somewhat different placement of electrodes. The rats in group B were also operated on and had electrodes inserted similarly but no electric current was passed. The remaining ten rats, which served as a different control group (group C), underwent no operation. Prior to experimental use, the rats were maintained on laboratory chow of vegetable origin (Simonsen Farms green diet) containing 59 mg (calculated as sitosterol) of Liebermann-Burchard positive substances per 100 gm of diet. Following operation and for two weeks thereafter, all rats, including unoperated controls, were allowed to drink 30 ml/day of a previously described high-cholesterol (650 mg/100 ml) liquid diet,[9] which each rat consumed completely each day. All rats were fed the high cholesterol liquid diet for two weeks. Plasma cholesterol level was determined at the end of the first and second week. After the second cholesterol assay, each rat was returned to the pellet diet ad lib over a period of three more weeks. Then they were injected subcutaneously with 10.0 ml of N-saline solution. Immediately after the injection, they were placed in individual metabolic cages and the urine excreted during the following 24-hour period was

collected without preservative. The urine volumes were 12.2 ± 7.2, 12.2 ± 8.4 and 11.9 ± 3.2 ml in the three groups, respectively. Each urine sample was stored frozen (-17°C) until it was processed for mass spectrometric analysis.

Rat urines were individually sampled and analyzed for their organic metabolites. The metabolites were isolated on a chromosorb P (Supelco Inc., Bellefonte, PA) column using dichloromethane as the eluting solvent, with the subsequent concentration and transfer into a miniature chromosorb P column.[28] The FIMS analysis was performed on a 35 cm 60° sector magnet mass spectrometer comprising a solid probe inlet, a glass-lined slit-type cobalt-activated field ionization source,[31,32] and a 4096-channel multi-scaler for data accumulation.[33] The mass range 90 to 310 amu was spanned. The integrated mass spectra were transferred to magnetic tape and then analyzed on a Burroughs 6700 computer.

Each spectrum was normalized so that the sum of all but the five largest peaks was equal to unity. This procedure avoids biasing the variances in favor of the variances due to a few dominant peaks.[26] Duplicate molecular weight distribution analyses were performed on each specimen. The normalized duplicate spectra were averaged, producing three groups of ten average spectra each. Because of anomalies in their cholesterol concentrations (measured independently), the spectra representing the urine extracts of two rats were omitted from the statistical training sets, resulting in nine spectra for Group A (hypothalamus-injured), ten spectra for Class B (sham-operated), and nine spectra for Class C (intact, control).

The statistical analysis of the data included a Wilcoxon test at each peak, followed by a WNI analysis comparing the individual weighted spectra to the weighted averages of class spectra.[29-34] To enhance interclass differences, the reciprocal of the p-value was chosen as a weighting factor in the WNI computations. For instance, for spectrum i compared to the average of group B,

$$WNI_i(B) = 100 \sum_{j=1}^{J} \left\{ 1/p_j \left[A_{ij} - \bar{A}_j(B) \right] / (A_{ij} + \bar{A}_j(B)) \right\} / \sum_{j=1}^{J} 1/p_j$$

where p_j is the probability derived for peak j from the Wilcoxon tests on groups A and B, and $\bar{A}_j(B)$ is the average value of the normalized area of peak j in Group B.

The difference $D_i = WNI_i(A) - WNI_i(B)$, previously used as the diagnostic parameter,[29] was transformed to D_i^*.

$$D_i^* = D_i - (1/2\bar{D}(A) + 1/2\bar{D}(B)/1/2\bar{D}(A) - 1/2\bar{D}(B)$$

where $\bar{D}(A)$ and $\bar{D}(B)$ are the average D_i scores for classes A and B respectively. This transformation normalizes the D_i values of an unknown to units of one-half the difference between the means of the reference calss D's, enabling us to arbitrarily choose zero as a decision threshold. The values $D^*(A) = +1$ and $D(B)^* = 1$ correspond to the average scores for the A and B classes, respectively. *Since* $D_i^* = 0$ *always falls between* the two class average scores, it can be used consistently as the diagnostic threshold. For example, if class B is the control group, $D_i > 0$ for spectrum i implies a diagnosis of this specimen as an abnormal one. A figure of merit, the "diagnostic power" (DP) is derived from the percentage of successful identifications resulting from the analysis of a pair of classes in a training set; where $0 \leq DP \leq 1$.

Results

The average weight (in grams ± standrad error) of the three groups of rats, in order of hypothalamus-injured, sham-operated, and non-operated were as follows: pre-operation 301 ± 19, 308 ± 7, and 300 ± 7; one week post-operation 270 ± 12, 292 ± 17 and 292 ± 5; two weeks post-operation 275 ± 4, 301 ± 4, and 301 ± 5; five weeks post-operation 313 ± 9, 346 ± 8, and 351 ± 3. Food intake was not measured during the 2-5 week interval while the rats were on an ad lib diet of laboratory chow of vegetable origin. The average serum cholesterol levels ± standard error in the

three groups were 99 ± 22, 61 ± 10, and 57 ± 8 mg/100, respectively, one week post-operation, and 122 ± 36, 61 ± 13 and 63 ± 17 mg/100 ml one week later. Seven of the ten hypothalamus-lesioned rats showed extreme elevations in cholesterol level (103 to 157 mg/100 ml) and the remaining three rats showed smaller increases (66 to 93 mg/100 ml). The rat with the 66 mg/100 ml was excluded from the statistical analysis of Group A. No differences were observed in cholesterol level between sham-operated and the intact control rats.[12,13] One control rat showed, however, an unaccountably high serum cholesterol level (148 mg/100 ml). This rat was, therefore, excluded from the initial statistical analysis of Group C.

A total of 60 samples, duplicates of each urine sample, were analyzed in a random order. Our method gave reproducible and comparable spectral profiles with 0.50 ml of urine, as demonstrated by Figure B-1. More than 200 peaks with molecular weights between 90 and 310 amu have been quantitatively measured.

An average spectrum was computed for each group, as shown in Figure B-2, and used in the Wilcoxon test computations of p-values for comparisons between the three possible pairs of classes. The $\bar{B}$ and $\bar{C}$ spectra are rather similar, whereas the $\bar{A}$ spectrum is readily distinguished, even by visual inspection, showing p. minent peaks as mass numbers 250 and 298.

The mass numbers and p-values for the most significant differences in each comparison are shown in columns 2 and 3 of Tables B-1, B-2, and B-3. The entire lists, consisting of 214 p values, are displayed logarithmically in Figure B-3. While the B-C differences show relatively low significance, the metabolites of 125, 127, 139, 144 and 160 amu also appear among the most significant differences in the A-C comparisons, indicating metabolic pertubations induced by the sham operation and detectable five weeks later. The most significant amu values in column 2 of Tables B-1 and B-2, e.g., 298, 250, Or 251 amu, indicate metabolites, the concentrations of which were altered by the hypothalamic lesion.

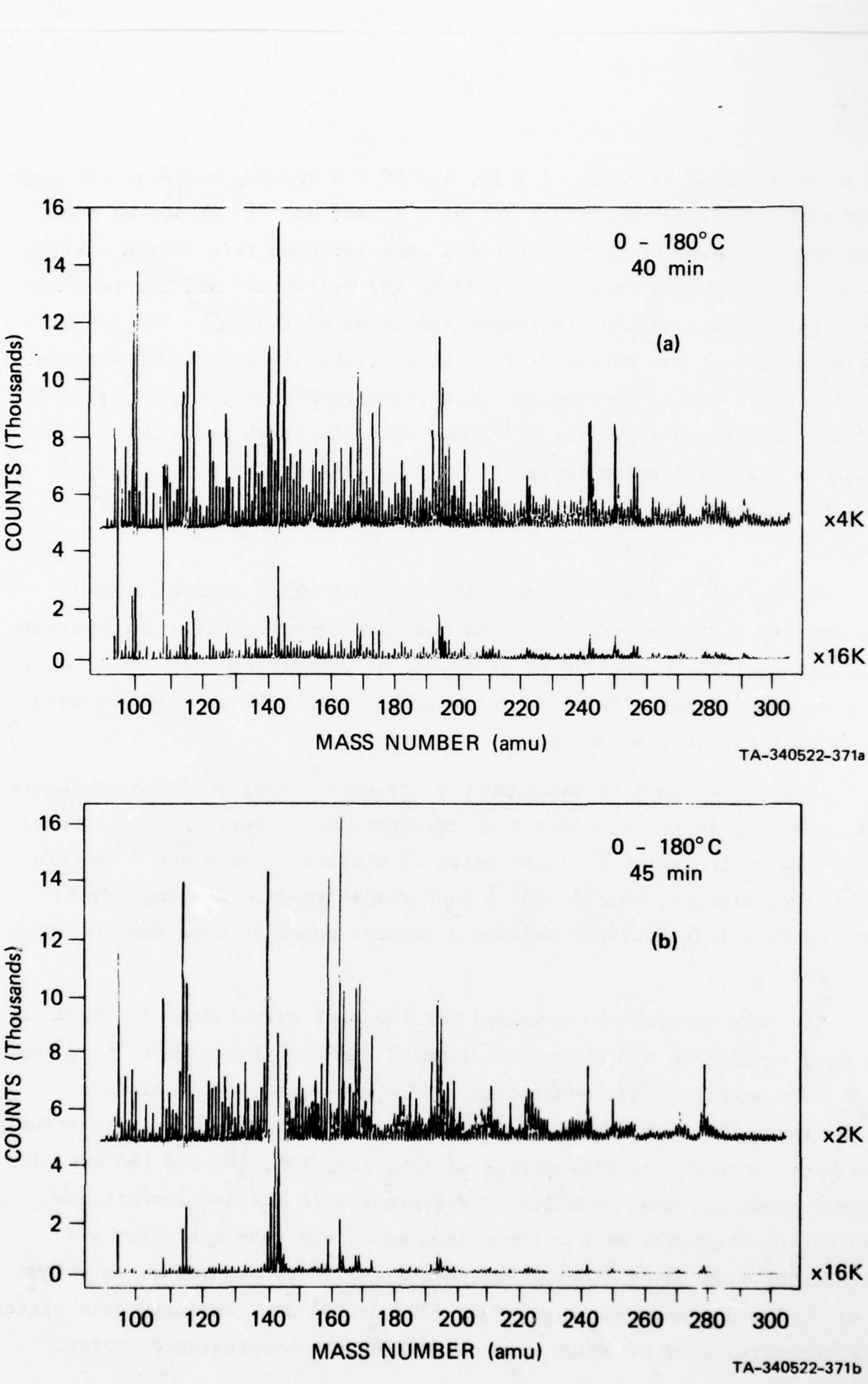

FIGURE B-1 TWO TYPICAL RAW SPECTRA OF RAT URINE

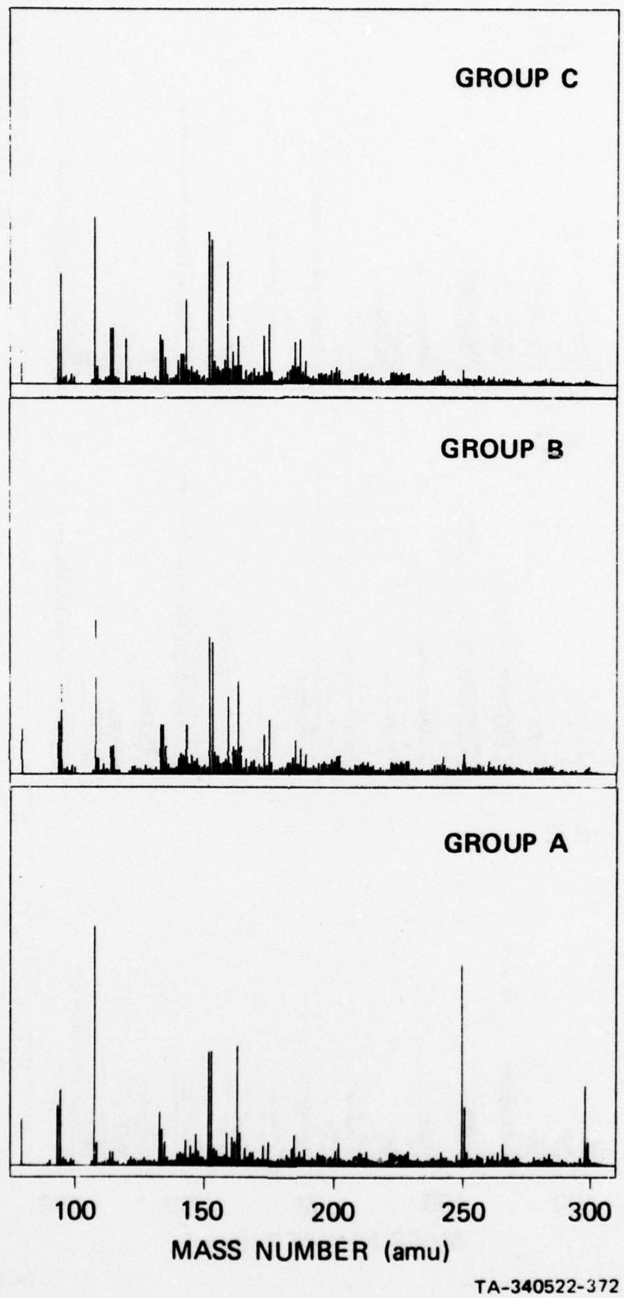

FIGURE B-2 AVERAGE NORMALIZED SPECTRA

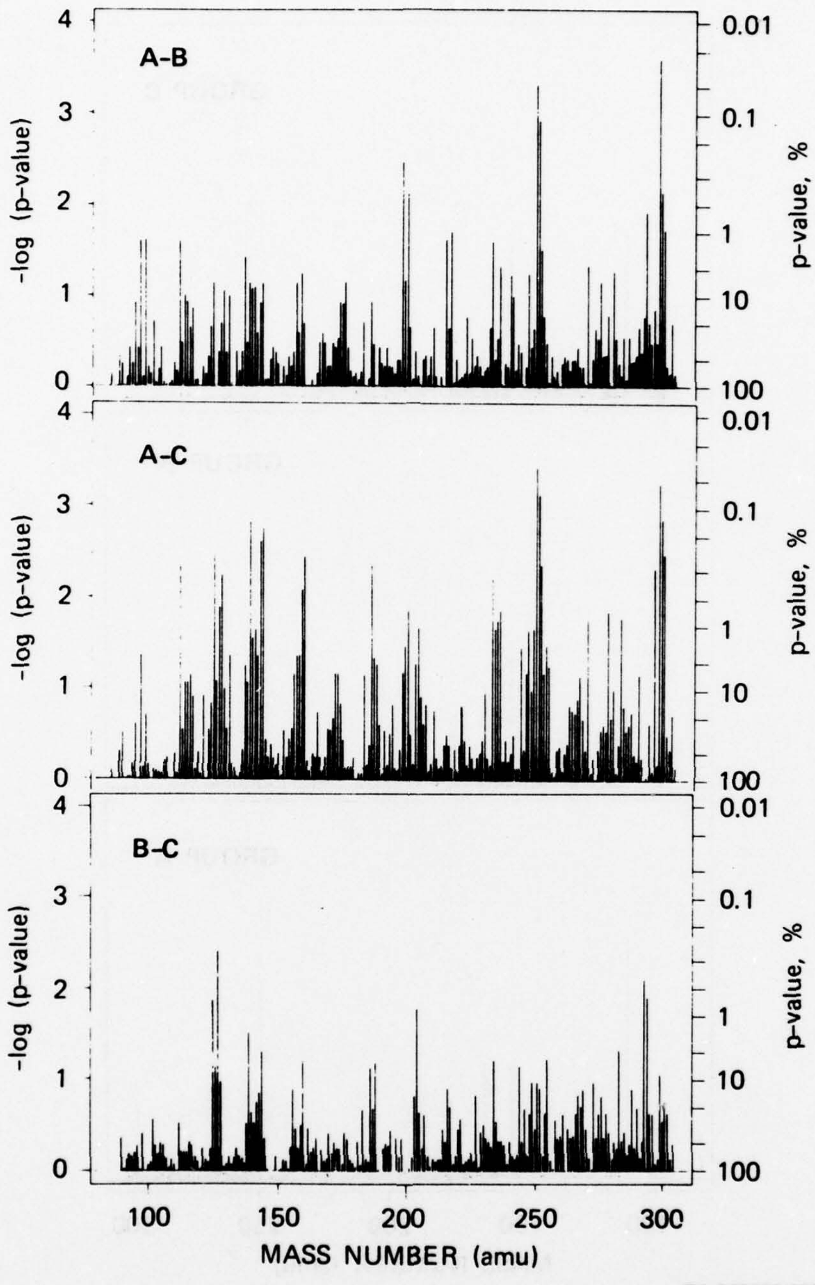

FIGURE B-3 P-VALUE SPECTRA FOR GROUP COMPARISONS

Table B-1. Diagnostic Power (DP) as a Function of p-values for A-B Comparisons over m_j to m_{j+3} and over First j Mass Numbers (DP*)

j	m	p%	DP%	DP*%
1	298	0.024	100	100
2	250	0.045	91.1	100
3	251	0.11	84.4	100
4	198	0.33	95.5	100
5	200	0.71	88.9	100
6	299	0.9	82.2	100
7	293	1.1	77.8	100
8	217	1.8	77.8	100
9	300	2.8	68.9	100
10	215	2.2	84.4	100
11	112	2.2	77.8	100
12	97	2.2	75.5	100
13	99	2.2	71.7	100
14	233	2.2	64.4	100
15	252	2.7	68.9	100
16	137	3.4	64.4	100
17	236	4.1	62.2	100
18	270	4.1	51.1	100
19	159	5.0	62.2	100
20	240	5.0	82.2	100
21	247	5.0	77.8	100
22	280	5.0	71.1	100
23	139	6.0	57.8	100
24	275	6.0	64.4	100
25	199	6.0	48.9	100
100	291	37.0	37.8	100
150	263	62.0	15.5	100

Table B-2. Diagnostic Power (DP) as a Function of p-values for A-C Comparisons over m_j to m_{j+3} and over First j Mass Numbers (DP*)

j	m	p%	DP%	DP*%
1	250	0.035	100	100
2	298	0.049	100	100
3	251	0.067	97.5	100
4	299	0.13	90.1	100
5	139	0.13	92.6	100
6	144	0.17	90.1	100
7	143	0.23	87.6	100
8	125	0.31	90.1	100
9	160	0.31	87.6	100
10	300	0.31	92.6	100
11	296	0.41	95.0	100
12	112	0.41	95.0	100
13	186	0.41	95.0	100
14	252	0.41	75.3	100
15	233	0.54	82.7	100
16	128	0.54	85.2	100
17	159	0.71	85.2	100
18	236	1.2	97.5	100

Table B-3. Diagnostic Power (DP) as Functions of p-values for B-C Comparisons over m_j to m_{j+3} and over First j Mass Numbers (DP*)

j	m	p%	DP	DP*%
1	127	0.33	88.9	80.0
2	293	0.71	80.0	84.4
3	125	1.1	84.4	86.7
4	294	1.1	77.8	88.9
5	204	1.4	80.0	86.7
6	139	2.7	73.3	88.9
7	283	4.1	84.4	88.9
8	144	4.1	75.5	91.1
9	255	5.0	71.1	91.1
10	234	5.0	75.5	91.1

Table B-4. Percent Identification of Numbers of a Given Group with Established Pattern of Groups A, B, or C

X	({$\bar{A}$},	{$\bar{B}$})	({$\bar{A}$},	{$\bar{C}$})	({$\bar{B}$},	{$\bar{C}$})
{A}	100,	0	100,	0	55,	45
{B}	0,	100	0,	100	90,	10
{C}	0,	100	0,	100	11,	89
#6	False	True	False	True	False	True
#10	False	True	False	True	False	True

The use of the reciprocal p-value as a weighting factor for the WNI computation tends to obscure the contribution of certain significant mass numbers, since the degree of diagnostic success will be determined, for the most part, by the spectral differences at a few mass numbers. It was decided, therefore, to compute the DP (using T = 0 as the decision threshold for the normalized D_i^*) in two ways. One was to use four peaks at a time. The j^{th} entries in column 4 of Tables B-1, B-2, and B-3 were computed using p_j to p_{j+3}. For comparison, the j^{th} entries in column 5 were computed using p_1 to p_j, where it can be seen that as a result of the use of the reciprocal p-value weighting function, the two classes can be separated with 100% success in spite of the fact that most of the peaks in the range $1 < j < 150$ have p-values greater than 5%.

Each individual spectrum was then compared to each of the three reference pairs at the mass numbers corresponding to the <u>four</u> smallest p-values (associated with the reference pair). Table B-4 was compiled using the paris A-C, A-B, and B-C as learning sets. Note that, in a comparison of the spectra in class B to the reference pair $\bar{A}$, $\bar{C}$, all of the spectra were identified with $\bar{C}$, reflecting the similarity between the $\bar{B}$ and $\bar{C}$ spectra in Figures B-2 and B-3. Similarly, all of the $\bar{C}$ spectra are identified with $\bar{B}$ in the $\bar{A}$,$\bar{B}$ pair. From Tables B-1, B-2, and B-3 it may be concluded that four metabolic parameters were sufficient to differentiate between the three groups. In fact, just two parameters, namely the metabolites with molecular weight 250 and 298 were sufficient to distinguish between groups A-C and A-B.

An interesting test of the methodology was the examination of the identity of the two rats which have been excluded from the original classification because of their "abnormal" cholesterol levels. As can be seen in Table B-4, Rat #6, an intact rat with an abnormally high cholesterol level was still identified as a member of Group C. In other words, its urine did not reflect any of the features associated with a hypothalamic lesion; probably in this case, by human error, no electric current was applied to the hypothalamus.

Discussion

It has been known that a chronic hypercholesterolemia is caused by the previously described selective lesion of the hypothalamus. The present work confirms this observation and indicates additional distinct metabolic disorders induced by this type of hypothalamus lesion. The metabolites, which have amu values revealed by the statistical analysis as a characteristic molecular weight pattern, may be considered as metabolic parameters associated with the hypercholesterolemia, which are detectable five weeks after the treatment. The observed differences in urinary metabolic profiles between the sham-operated and the intact controls indicate metabolic changes induced by the operation, distinct from those induced by the hypothalamus lesion.

The demonstration of significant differences in the concentrations of certain metabolites in urine that underwent a certain pretreatment, makes it highly likely that additional metabolic aberrations will be detectable in the plasma or in urine when pretreated differently (e.g., extracting the acidic metabolites).

The chemical structures of the metabolites that contributed to the significant peaks in the molecular weight profiles are yet to be determined. This may be accomplished by the collision-induced fragmentation technique.[35] Prior to such an analysis any assignment of chemical identity to these parameters would be highly speculative.

Although just two parameters are sufficient to distinguish between the rats with lesions and their controls, the real value of our methodology is the identification of many additional metabolites, the concentration of which is significantly altered by the treatment. The metabolic implications and biochemical origins of the compounds of interest could provide information on the regulatory functions of the hypothalamus, as well as on the biochemical implications of the damage induced by our kind of treatment.

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APPENDIX C

Diagnostic Patterns of Metabolites in Urines of Patients With Pulmonary and Urinary Infections

Summary

Organic metabolites with low molecular weights (between 86 and 306 atomic mass units) were determined in urines from patients with different infectious diseases, especially urinary and pulmonary infections. The analytical method is based on a rapid column chromatographic isolation of metabolites from urine, field ionization mass spectrometric analysis of the metabolites to obtain molecular weight profiles, and computer statistical analysis of the profiles to extract diagnostic information.

Introduction

Biochemical abnormalities have been extensively analyzed in patients suffering from different infectious diseases.[1] In an attempt to identify metabolic changes associated with acute infectious hepatitis, low molecular weight organic metabolites present in urine have been studies using field ionization mass spectrometry (FIMS).[2] The results have indicated that infectious hepatitis can be diagnosed with a high degree of confidence through analysis of molecular weight profiles of the acidic or neutral metabolites in urine.

In the biomedical field, rapid screening of biological fluids has been accepted as a useful aid for the diagnosis of hereditary as well as other metabolic disorders. This has been successfully achieved by a combination of gas chromatography, mass spectrometry, and computer analysis.[3-11] Because of its nonfragmenting nature, FIMS is ideally suited for multicomponent analysis of complex biological samples without prior chromatographic separation.[12] Following our preliminary work on infectious hepatitis,[2] we have simplified the preseparation techniques

and improved the mass spectrometric instrumentation, while maintaining low coefficients of variation.[13] These developments, as well as improvements in the statistical data analysis,[14] make FIMS methodology even more attractive.

As a continuation of the study on infectious diseases, urine samples of patients suffering from infectious diseases other than infectious hepatitis were examined employing the improved methodology. The results reported in this paper are highly encouraging, since they indicate that this procedure may be of diagnostic value.

Materials and Methods

Reagents. Methylene chloride, reagent grade, (Mallinkrodt Chemical Works, St. Louis, MO, USA) was distilled.

Sodium chloride, Baker Analyzer Reagent was obtained from Baker Chemical Co., Phillipsburg, NH, USA.

Adsorbent. Chromosorb P, 80/100 mesh, acid washed, (Supelco Inc., Bellefonte, PA, USA) was used for both isolation column and concentration column materials.

Apparatus. Figure C-1 presents the sampling system used for isolation and concentration of organic metabolites from urine samples. Columns were prepared and conditioned as described in Appendix A.

Sampling Procedure. Urine samples were obtained from the University Hospital, University of California Medical Center, San Diego. The 15 patients included 6 with confirmed urinary infections, 1 acute peritonitis and acute urinary infection, 2 active pulmonary tuberculosis, 2 acute bronchitis, 1 acute salpingitis, 1 acute pneumococcal pneumonia, 1 acute sinusitis, and 1 salmonella typhi infection. Control urine samples were collected from 15 healthy volunteers. All urine samples were kept in a freezer (-17°C) until they were individually sampled for their organic metabolites.

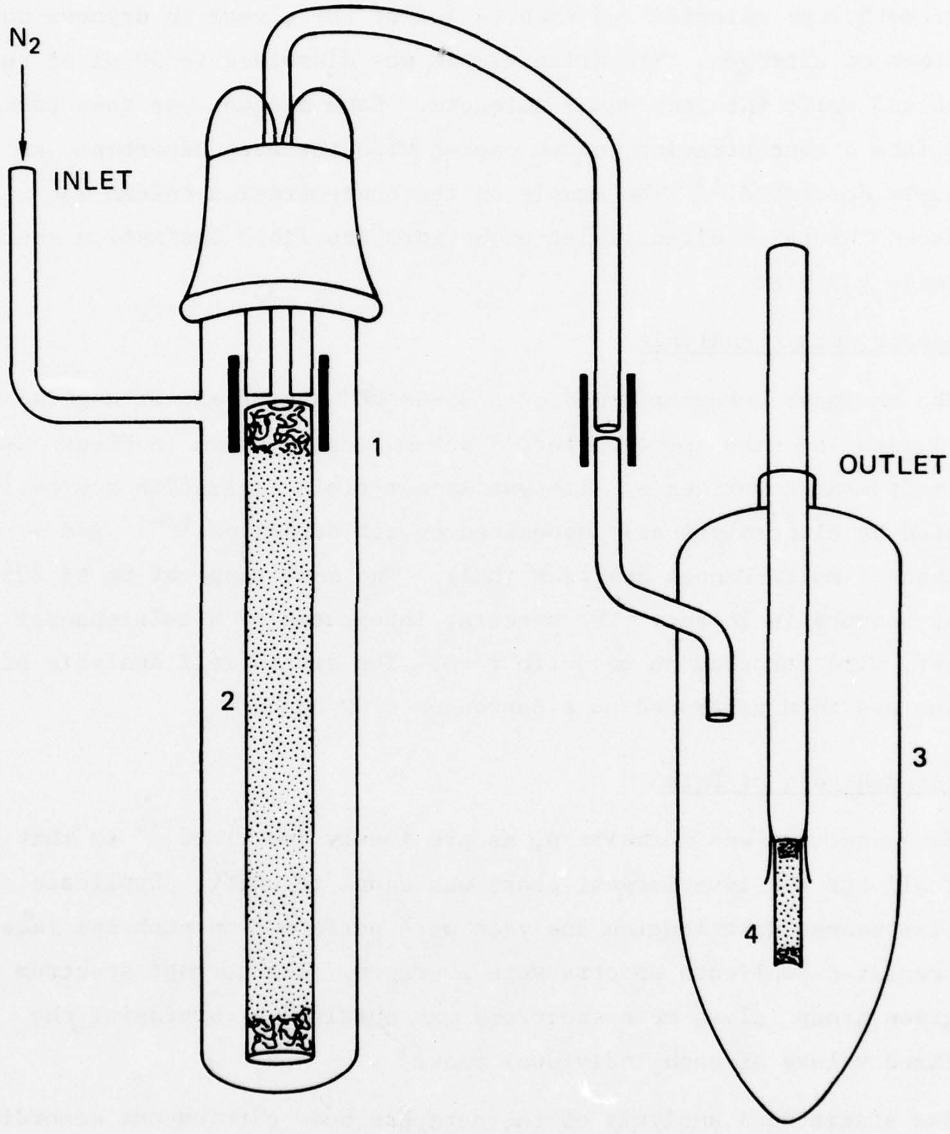

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FIGURE C-1 SAMPLING SYSTEM: (1) ISOLATOR, (2) ISOLATION COLUMN, (3) CONCENTRATOR (4) CONCENTRATION COLUMN, (5) TEFLON TUBING

Using the apparatus shown in Figure C-1, 1 ml of urine to which 0.3 g of sodium chloride was added, was adsorbed onto the isolation column packed with chromosorb P, followed by elution of the metabolites with 5 ml of methylene chloride and evaporation of the eluate to dryness under the stream of nitrogen. The dried sample was dissolved in 50 μl of the solvent and split into two equal aliquots. Each aliquot was then transferred into a concentration column packed with the same adsorbent, as previously described.[13] The sample on the concentration column was introduced through a direct inlet probe into the field ionization source to be mass analyzed.

Mass Spectrometric Analysis

The analyses were performed on a 35-cm 60° sector magnet multiscanning field ionization mass spectrometer,[13] schematically shown in Figure C-2. This instrument comprises a slit-type linear field ionization source,[16] activated by electrolytically deposited cobalt dendrites,[13-17] and a 4096-channel multichannel analyzer (MCA). The mass range of 86 to 306 amu was scanned in 16 sec. The spectra, integrated by a multichannel analyzer, were recorded on magnetic tape. The statistical analysis of the data was then perfromed on a Burroughs 6700 computer.

Computer Analysis of Data

Each spectrum was normalized, as previously described,[14] so that the sum of all but the five largest peaks was equal to unity. Duplicate molecular weight distribution analyses were performed on each specimen. The normalized duplicate spectra were averaged. The average spectrum of a given group (class mean spectrum) was obtained by averaging the normalized values of each individual peak.

The statistical analysis of the data has been carried out according to the procedure described in Appendix B.

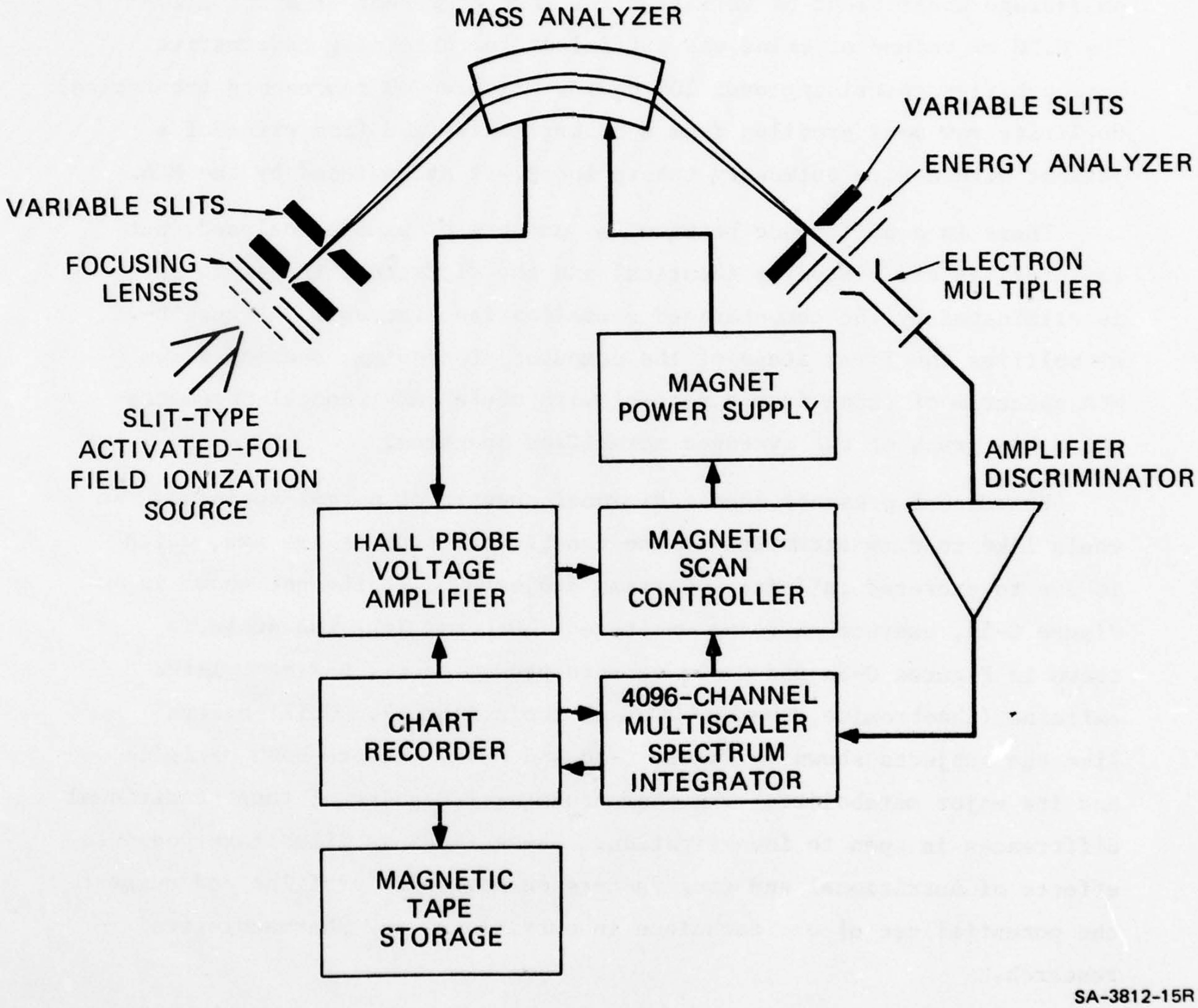

FIGURE C-2 SCHEMATIC DIAGRAM OF MULTISCANNING FIELD IONIZATION MASS SPECTROMETER

Results and Discussion

Each of 30 urine samples from 15 patients and 15 healthy subjects (controls) was analyzed in two separate runs. The technique involves less than two hours for sample preparation and mass spectrometry, yielding an average coefficient of variation for each component of about 11%.[13] The 0.50 ml volume of urine was sufficient for obtaining informative mass profiles comprising over 200 peaks. Figure C-3 represents the typical duplicate raw mass profiles from a control urine and from urine of a patient with active pulmonary tuberculosis, as accumulated by the MCA.

There is a difference between the amounts of sample analyzed, but the profiles are virtually identical and the difference in sample size is eliminated by the computerized normalization procedure. Figure C-4 exemplifies the first stage of the computer processing, showing a raw MCA spectrum of urine from a patient with acute pneumococcal pneumonia and a bar graph of the averaged normalized spectrum.

Figure C-5 presents four additional spectra of normal subjects. We would like to draw attention to the conspicuous peak at 194 amu, which is due to excreted caffeine. Certain subjects, like the one shown in Figure C-5a, excrete caffeine unaltered. Others, like the subjects shown in Figures C-3a and C-5b, excrete predominantly N-dimethylated caffeine (theobromine, theophylline, or their isomer). Still others, like the subjects shown in Figure C-5c and C-5d, excrete both caffeine and its major metabolite. The pharmacological meaning of these individual differences is open to investigation. These findings illustrate possible effects of nutritional and drug factors on metabolic profiles and suggest the potential use of our technique in nutritional and pharmacological research.

Figure C-6 shows a number of representative raw MCA spectra of four patients. The most conspicuous difference between the controls and the patients is the absence of caffeine and its demethylated metabolite from the urine of hospitalized patients. This may not be surprising, but it points out that the appearance of major metabolites at 194 and 180 amu does not necessarily indicate good health. To state it more scientifically,

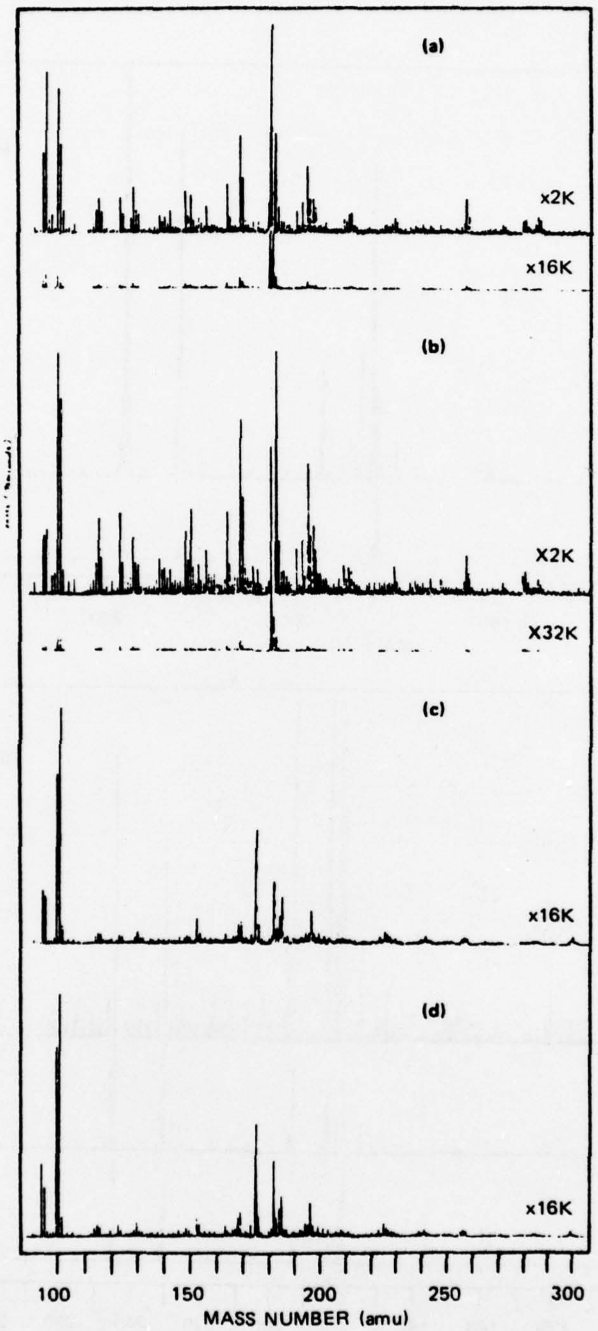

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FIGURE C-3 REPRESENTATIVE DUPLICATE MCA SPECTRA OF NORMAL URINE (a) AND (b), AND OF URINE OF A PATIENT WITH PULMONARY TUBERCULOSIS (c) AND (d)

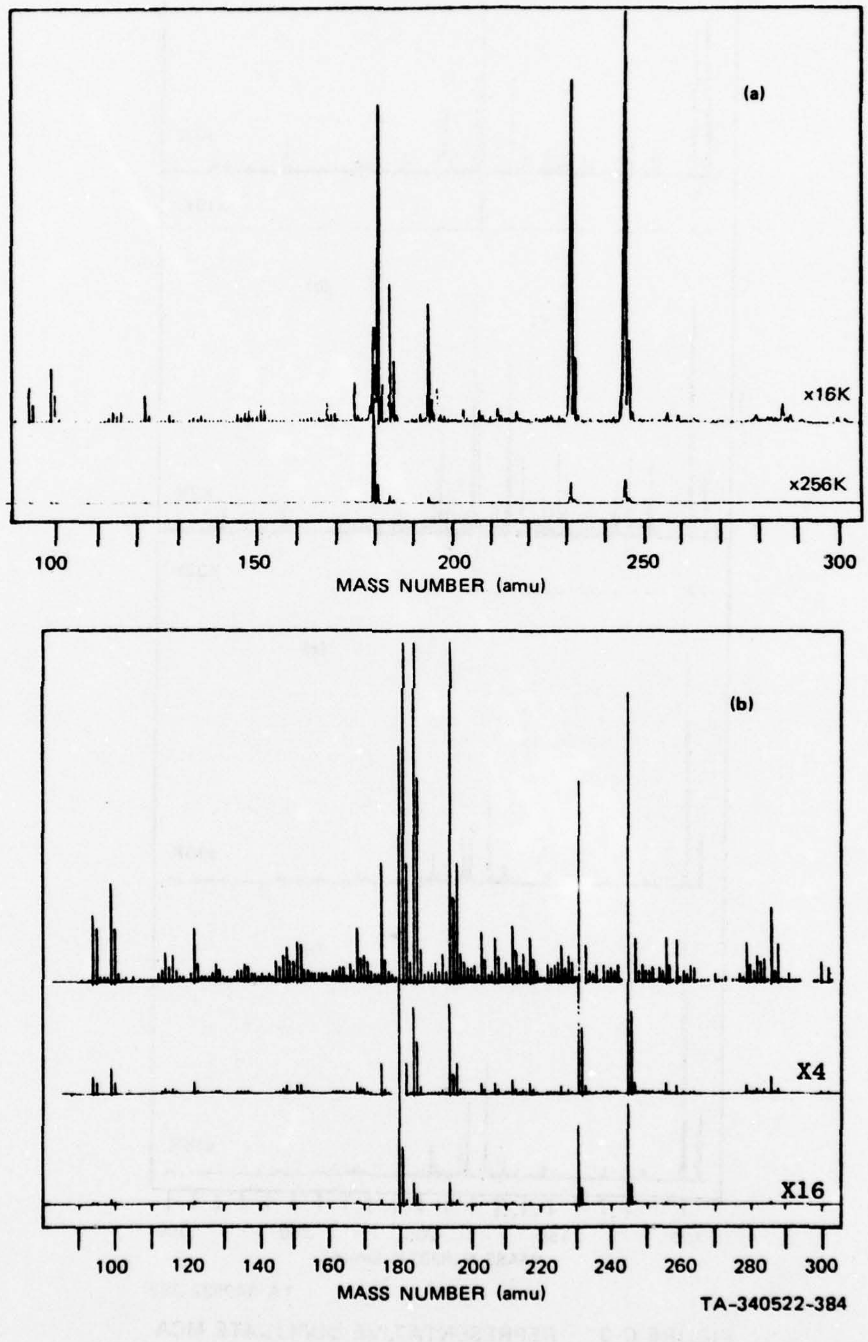

FIGURE C-4 (a) MCA AND (b) COMPUTER NORMALIZED AVERAGE SPECTRUM OF DUPLICATE ANALYSIS OF URINE OF A PATIENT WITH PNEUMOCOCCUS PNEUMONIA

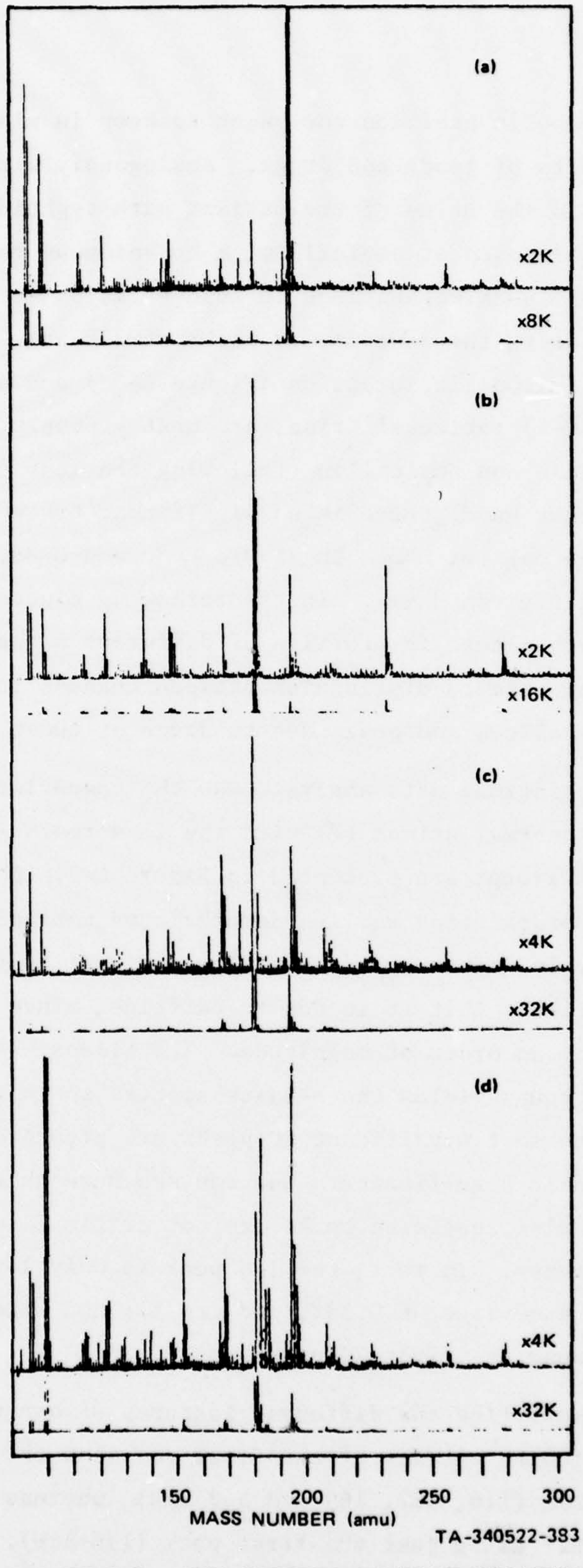

FIGURE C-5 MCA SPECTRA OF FOUR HEALTHY INDIVIDUALS

when analyzing metabolic profiles one ought to keep in mind and discount the potential effects of foods and drugs. Analogously with the case of caffeine, we find in the urine of the patient with typhoid fever, who obtained substantial doses of ampicillin, a conspicuous peak at 151 amu, due most probably to α-aminophenylacetic acid--a major metabolite of the antibiotic. Similarly, the conspicuous peaks at 232 and 246 amu, which are common to the salmonella infection (Figure C-6c) and pneumococcus pneumonia (Figure C-4) patients' urine, are most probably due to metabolites of penicillin and ampicillin (following the loss of the side chain). On the other hand, there is no significant trace of ethambutal in the urine of the patient shown in Figure C-3c and C-3d, although 200 mg of this drug were given daily. In the following discussion, when we consider the urinary metabolic profiles of different patients, we shall try to take special care to distinguish between changes in the concentrations of body metabolites and peaks due to drugs or their metabolites.

The first statistical data analysis was the comparison of the total population of 15 abnormal urines (P) with the 15 normals (C). The average spectra of the two groups are presented in Figure C-7. Note again the conspicuous peaks of caffeine and its demethylated metabolite at 194 and 180 amu, respectively. The P group has a predominant component at 180 amu, but it is unlikely that it is due to caffeine, since the peak at 194 amu is lower by an order of magnitude. The Wilcoxon test on all the peaks of the two groups yields the p-value spectra shown in Figure C-8, and the data of the most significant 20 peaks are presented in Table C-1 in the order of their significance. One can see here that in spite of their outstanding size, caffeine peaks are not critical in distinction between the two groups. In fact, the 180 peak is only 17th in order of significance with a p-value of 0.58%, and its 181 amu satellite peak does not even appear among the first 20 peaks.

Table C-1 exemplifies the different features of our statistical analysis.[15] The fourth entries of the first row show the DP values using the first four peaks (116, 127, 169 and 168 amu), whereas the fifth entries show the DP* using just the first peak (116 amu). As we go to the second row, we obtain the DP using peaks 127, 169, 168, and 194 and

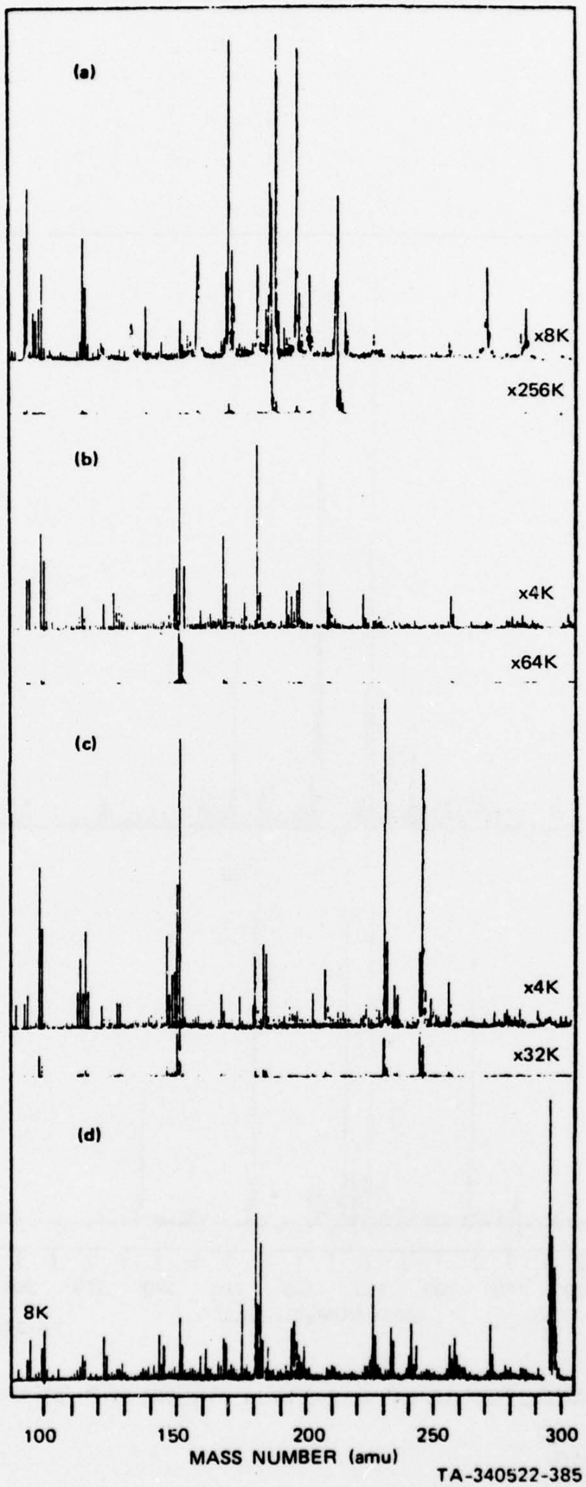

FIGURE C-6 MCA SPECTRA OF URINES FROM CASES OF (a) ACUTE SINUSITIS (b) ACUTE URINARY INFECTION (c) SALMONELLA AND (d) ACUTE URINARY INFECTION PLUS ACUTE PERITONITIS

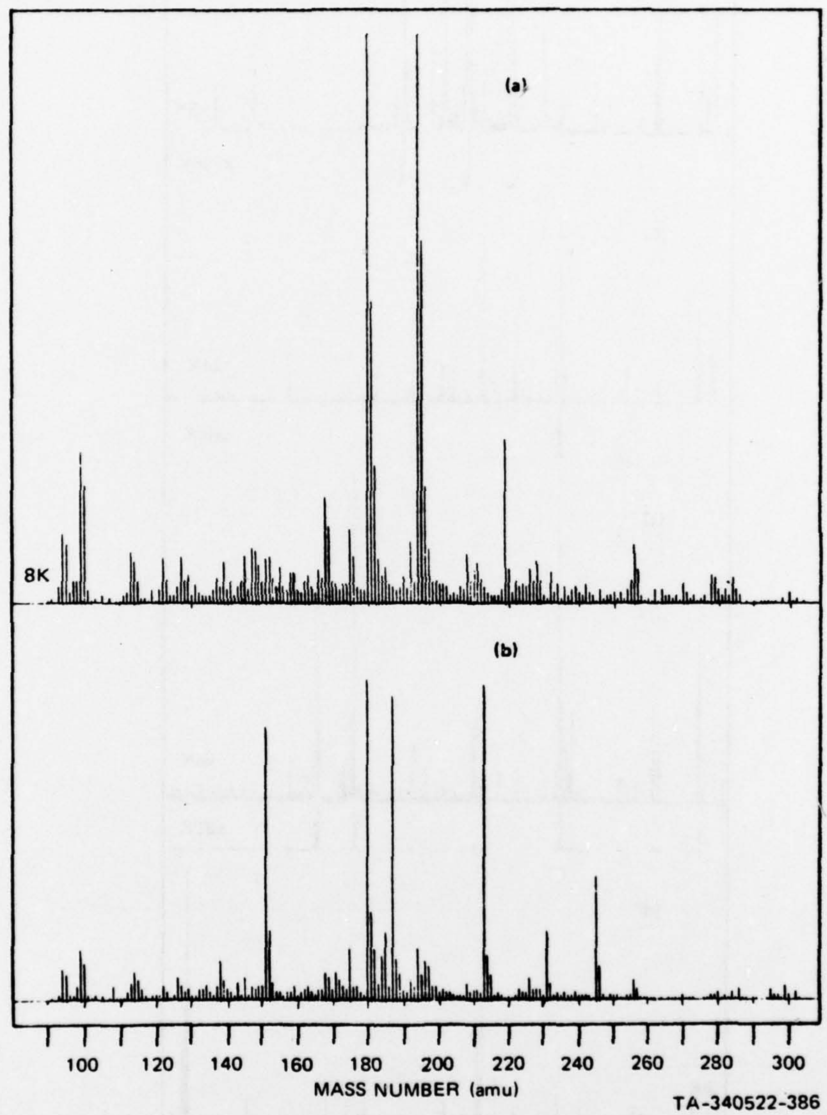

FIGURE C-7 AVERAGE SPECTRA OF (a) NORMAL GROUP AND (b) ABNORMAL GROUP

DP* using the first two peak (116 and 127 amu). The third row shows the DP using peaks 3 through 7 (169, 168, 194 and 195 amu) and DP* using the first 3 peaks (116, 127 and 169 amu), and so on. As we go down the table, the values of DP* increase as more peaks are used in the diagnostic test. The increase is slow, however, because we use the reciprocal of p as the weighting factor in the analysis, thus the peaks with large p values contribute very little to the diagnostic power. Still, we can achieve a 93% successful diagnosis using all the peaks.

The fourth column is also very instructive, since it shows which peaks make the greatest contribution to the differentiation, which is not necessarily identical with the order of the p-values. Thus, for instance, the peaks of 155, 118, 117 and 177 amu (12th through 15th in the order of p-values, with DP = 78.7%) allow a more effective differentiation than the peaks of 194, 195, 211 and 185 amu (5th through 8th in the order of p-values, with DP = 62.7%--these coincidentally contain the caffeine peaks). This difference means that the former four peaks comprise a more powerful diagnostic combination than the latter four peaks, two of which are predominantly of exogenous origin.

The next case to be considered is a statistical analysis of the subgroups of seven patients suffering from urinary infections and five patients suffering from pulmonary infections. The average spectra of the group of urines from urinary infections (U) is given in Figure C-9a and that of the pulmonary infection group (PU) is presented in Figure C-9b. Figure C-9c is the average spectrum of the urinary profiles of the eight subjects who did not suffer from urinary infections (NU), including the members of the PU group.

Figure C-10a shows the p-value spectrum for the U-C comparison, and Figures C-10b and C-10c are the analogous figures for the PU-C and NU-C comparisons. Comparison between Figure C-10a and Figure C-8 shows less significant p-values in the U-C versus the P-C comparison--there are only 12 peaks with $p < 1\%$ as compared with 31 peaks of the P-C spectrum. As can be seen in Table C-2, this decrease in p-values does not diminish the diagnostic power.[15] Once can see that the urinary infection can be diagnosed with greater confidence using either four peaks at a

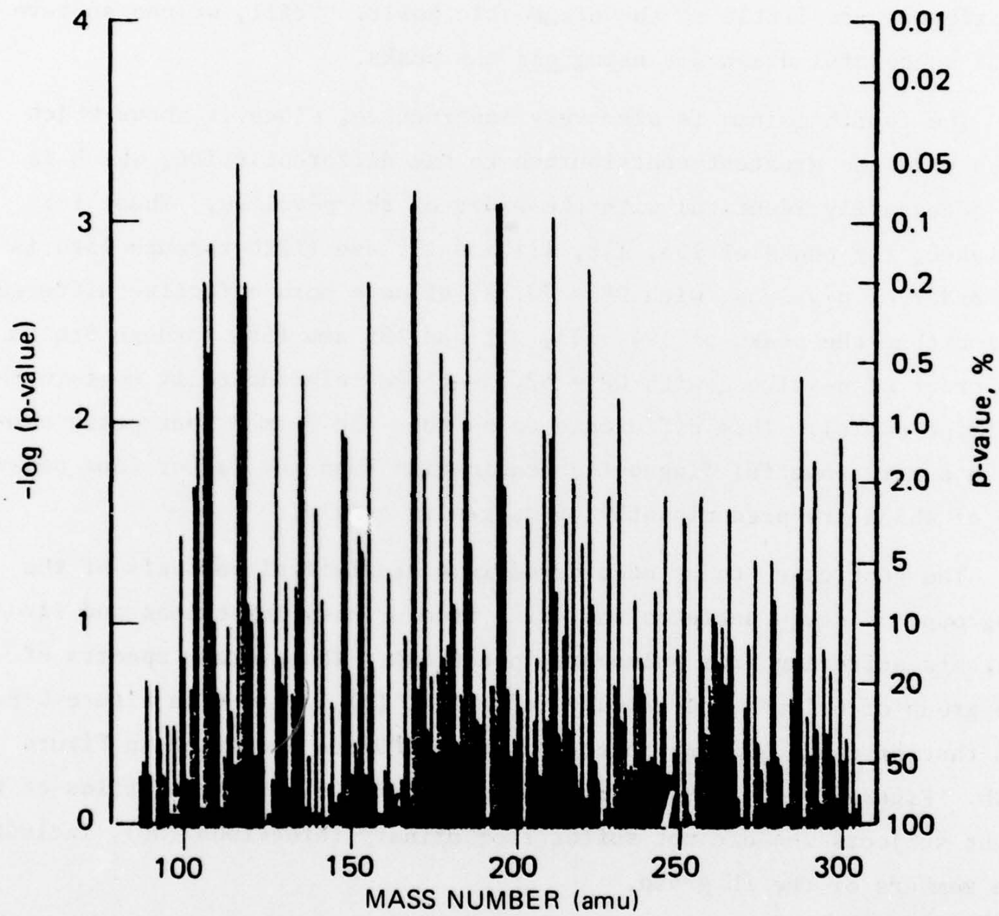

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FIGURE C-8 P-VALUE SPECTRA OBTAINED FROM NORMAL VERSUS COMBINED ABNORMAL GROUP COMPARISON

Table C-1. Diagnostic (DP) as a Function of p-Values for P-C Comparisons over m_j to m_{j+3}, and over First j Mass Numbers (DP*)

j	m	p%	DP%	DP*%
1	116	0.053	87.5	60.0
2	127	0.072	88.4	75.1
3	169	0.072	76.0	82.2
4	168	0.084	68.9	87.5
5	194	0.084	62.7	91.1
6	195	0.084	74.2	91.1
7	211	0.098	70.7	91.2
8	185	0.13	64.4	92.0
9	108	0.13	55.6	92.2
10	222	0.17	77.8	92.4
11	106	0.20	76.9	92.4
12	155	0.20	78.8	92.6
13	118	0.20	61.8	92.6
14	117	0.23	74.2	92.9
15	177	0.45	55.6	92.9
16	107	0.45	57.3	92.9
17	180	0.58	64.4	92.9
18	135	0.58	56.4	92.9
19	287	0.58	62.7	92.9
20	231	0.75	62.7	92.9
30	172	1.70	74.2	92.9
50	122	5.90	51.1	92.9
100	264	21.00	2.2	92.9

time or all the peaks (DP* = 100%) than can the heterogeneous population of patients with a variety of infectious diseases. It should be also noted that the peak corresponding to septazine (262 amu), frequently used in the treatment of urinary infections, does not appear conspicuously in Figure C-9a or in Figure C-10a, and it does not contribute an artifactual distinction in the U-C comparison.

The PU-C comparison shows six peaks with $p < 1\%$. As shown in Table C-3, these urines can be correctly diagnosed with approximately 95% probability, which is better than that achieved for the larger but more heterogeneous population of P. The last, NU-C, comparison, although showing 11 peaks with $p < 1\%$, can be diagnosed with less certainty than the larger heterogeneous population, as can be seen in Table C-4. Also of interest are the comparisons of the U-PU and U-NU groups. The results shown in Table C-5a and C-5b indicate outstanding diagnostic differentiation between the two groups of disorders.

Finding characteristic metabolic patterns associated with pathological conditions encourages the development of appropriate mass spectrometric techniques for the establishment of the chemical identity of those metabolites that show significant changes in concentration under pathological conditions. This could be invaluable information for the understanding of the etiology of diseases and for optimizing therapy. The first step in this direction has been the development of the high transmittance collisional fragmentation mass spectrometer[19] which allows the chemical identification of individual constituents by the fragmentation pattern of mass separated primary ions.

The results discussed above show that in spite of the limited number of patients examined, it was possible to demonstrate a diagnostic pattern for bacterial urinary infections (as a class) and probably for bacterial pulmonary infections (as another class). These preliminary results on two groups of infectious diseases corroborate our conclusion from the preliminary investigation of infectious hepatitis,[2] that the FIMS multicomponent analysis methodology is extremely powerful and should be explored further in greater detail as a tool for differential diagnosis of infectious diseases.

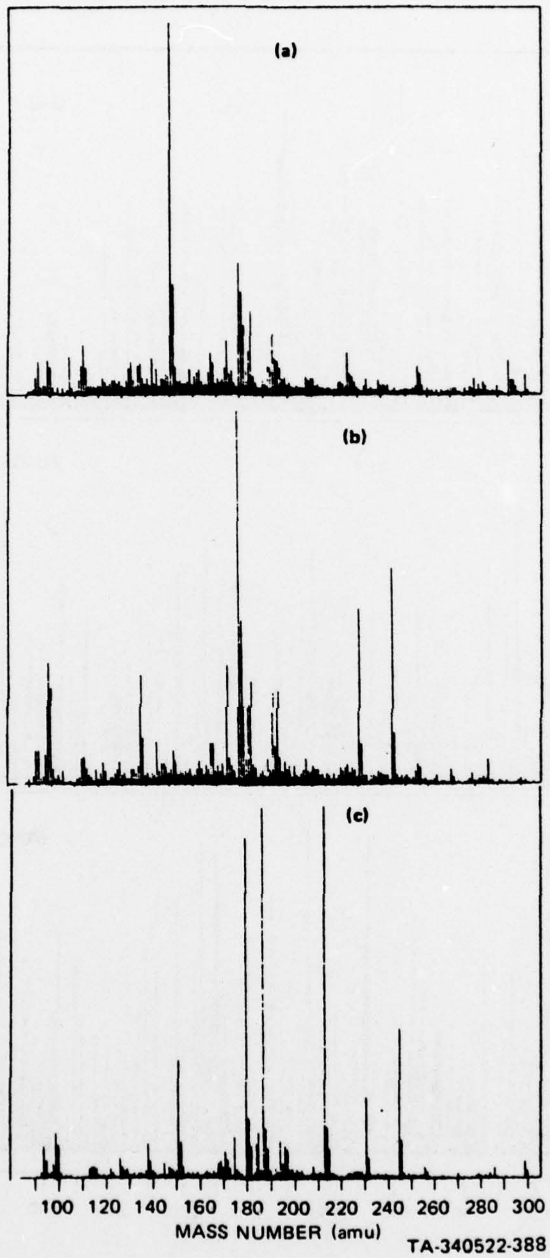

FIGURE C-9 AVERAGE SPECTRA OF ABNORMAL URINES FROM (a) URINARY INFECTION, (b) PULMONARY INFECTION, AND (c) COMBINED GROUP OF NONURINARY INFECTION

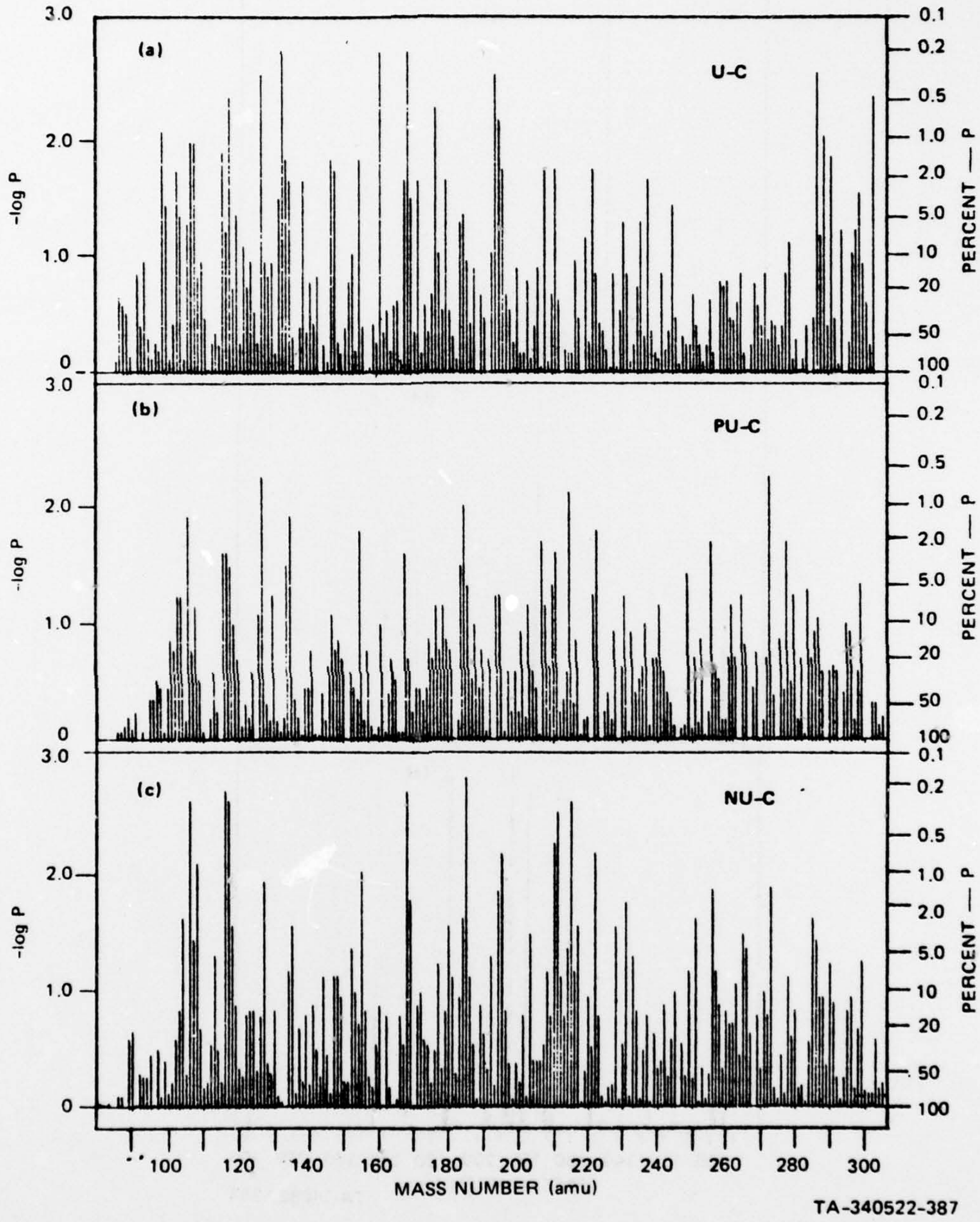

FIGURE C-10 p-VALUE SPECTRA OF (a) NORMAL VERSUS URINARY INFECTION (b) NORMALS VERSUS PULMONARY INFECTION AND (c) NORMAL VERSUS COMBINED NONURINARY INFECTION

Table C-2. Diagnostic Power (DP) as a Function of p-values for U-C Comparisons over m_j to m_{j+3} and over First j Mass Numbers (DP*)

j	m	p%	DP%	DP*%
1	133	0.17	96.2	
2	161	0.17	96.2	88.6
3	169	0.17	98.1	90.5
4	287	0.27	94.3	96.2
5	194	0.27	98.1	98.1
6	127	0.27	100.0	100.0
7	118	0.43	96.2	100.0
8	303	0.43	84.8	100.0
9	177	0.54	79.0	100.0
10	195	0.66	77.1	100.0
11	99	0.82	84.7	100.0
12	289	0.91	54.3	100.0
13	108	1.0	60.0	100.0
14	107	1.0	92.4	100.0
15	116	1.2	94.3	100.0

Table C-3. Diagnostic Power (DP) as a Function of p-values for PU-C Comparisons over m_j to m_{j+3}, and over First j Mass Numbers (DP*)

j	m	p%	DP%	DP*%
1	273	.597	92.0	30.7
2	127	.597	89.3	70.7
3	215	.776	81.3	86.7
4	185	1.022	84.0	92.0
5	135	1.286	73.3	89.3
6	106	1.286	86.7	89.3
7	223	1.638	97.3	92.0
8	155	1.638	94.7	92.0
9	207	2.072	94.7	92.0
10	256	2.072	86.7	92.0
11	278	2.072	94.7	92.0
12	211	2.603	86.7	92.0
13	168	2.603	84.0	92.0
14	116	2.603	68.0	92.0
15	117	2.603	78.7	92.0
16	184	3.247	78.7	94.7
17	134	3.247	76.0	94.7
18	118	3.247	76.0	94.7
19	249	4.024	78.7	94.7
20	210	4.953	73.3	94.7

Table C-4. Diagnostic Power (DP) as a Function of p-values for NU-C Comparisons over m_j to m_{j+3}, and over First j Mass Numbers (DP*)

j	m	p%	DP%	DP*%
1	185	0.16	88.3	67.1
2	168	0.19	93.3	75.0
3	116	0.19	88.3	75.0
4	106	0.24	86.7	88.3
5	117	0.24	86.7	90.0
6	215	0.24	86.7	90.0
7	211	0.30	81.7	90.0
8	210	0.55	80.0	90.0
9	195	0.67	81.7	90.0
10	222	0.67	83.3	90.0
11	108	0.81	76.7	90.0
12	155	0.98	73.3	90.0
13	127	1.2	66.7	90.0
14	273	1.3	68.3	90.0
15	256	1.4	85.0	90.3

Table C-5a. Diagnostic Power (DP) as a Function of p-values for U-PU Comparisons over m_j to m_{j+3}, over First j Mass Numbers (DP*)

j	m	p%	DP%	DP*%
1	133	0.74	94.3	94.3
2	124	1.18	100.0	100
3	298	1.85	83.3	100
4	297	2.84	77.1	100
5	161	2.84	100	100
6	215	2.84	100	100
7	148	2.84	100	100
8	207	2.84	100	100
9	107	4.24	100	100
10	100	6.18	71.4	100
11	200	6.18	82.8	100
12	235	6.18	77.1	100

Table C-5b. Diagnostic Power (DP) as a Function of p-values for U-NU Comparisons over m_j to m_{j+3}, and over First m Mass Numbers (DP*)

j	m	p%	DP%	DP*%
1	161	0.55	100.0	
2	124	0.78	89.3	92.9
3	215	1.1	71.4	100.0
4	133	1.1	64.3	100.0
5	289	2.1	57.4	100.0

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